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Immunogenicity and Ability of Variable Loop-Deleted Human Immunodeficiency Virus Type 1 Envelope Glycoproteins to Elicit Neutralizing Antibodies

Young B. Kim, Dong P. Han, Carlos Cao, and Michael W. Cho¹

Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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It has been extremely difficult to elicit broadly cross-reactive neutralizing antibodies (Nabs) against human immunodeficiency virus type 1 (HIV-1). In this study, we compared the immunogenic properties of the wild-type and variable loop-deleted HIV-1 envelope glycoproteins. Mice were immunized with recombinant vaccinia viruses expressing either the wild-type or the variable loop-deleted (V1-2, V3, V4, and V1-3) HIV-1_{env} gp160s. The animals were subsequently boosted with respective recombinant gp120s. All envelope constructs elicited similar levels of gp120-binding antibodies when analyzed by enzyme-linked immunosorbent assay (ELISA). However, the highest neutralizing activity was observed in sera from animals immunized with the wild-type envelope protein, followed by those immunized with Δ V4 and Δ V1-2. No neutralizing activity was detected in sera from animals immunized with Δ V3 or Δ V1-3. To identify immunogenic epitopes, ELISA was performed with overlapping 15-mer peptides that cover the entire length of gp120. For the wild-type gp120, the immunogenic epitopes mapped primarily to the variable loops V1-2 and to the conserved regions C1 and C5. When they were plotted onto known coordinates of gp120 core crystal structure, the epitopes in the conserved regions mapped predominantly to the inner domain of the protein. By immunizing with variable loop-deleted envelopes, the immune responses could be redirected to other regions of the protein. However, the newly targeted epitopes were neither on the exposed surface of the protein nor on the receptor binding regions. Interestingly, the removal of the V3 loop resulted in loss of immunoreactivity for both V3 and V1/V2 loops, suggesting structural interaction between the two regions. Our results suggest that obtaining broadly reactive Nabs may not be achieved simply by deleting the variable loops of gp120. However, the observation that the immune responses could be redirected by altering the protein composition might allow us to explore alternative strategies for modifying the antigenic properties of HIV-1 envelope glycoprotein. © 2002 Elsevier Science (USA)

INTRODUCTION

It is estimated that there are now over 40 million people infected with human immunodeficiency virus type 1 (HIV-1) worldwide (UNAIDS/WHO Report, 2001). Although it has been nearly two decades since HIV-1 was identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS), there is still no vaccine against the virus. With high cost and serious side effects of anti-retroviral therapy and the increase in viral resistance against anti-HIV-1 drugs, there is a great urgency for developing a vaccine against the virus.

One of the biggest obstacles in developing an effective AIDS vaccine is the extreme difficulty in eliciting neutralizing antibodies (Nabs) that are broadly reactive against many HIV-1 isolates that exist. The difficulty in eliciting Nabs against the virus comes from three major factors. First, the antigenic structure of the viral surface envelope glycoprotein, gp120, is highly variable from

isolate to isolate, with five hypervariable regions (V1–V5). Nabs directed against these variable regions are strain-specific, with very little cross-reactivity between clinical isolates. Second, gp120 is extensively glycosylated. There are 23 to 24 asparagine (N)-linked glycosylation sites and the carbohydrate moieties account for approximately one-half of the protein's entire mass. In addition to being important for the functional and structural integrity of the protein (see Ogert *et al.*, 2001 and references therein), these sugar residues affect the antigenicity and immunogenicity of the protein (Back *et al.*, 1994; Davis *et al.*, 1990; Huang *et al.*, 1997; Papandreou and Fenouillet, 1998; Reitter *et al.*, 1998; Schonning *et al.*, 1996). The carbohydrate structures, in effect, shield the virus from Nabs. Finally, the conserved regions of the protein that bind cellular receptors CD4 and coreceptors (viz. CXCR4 or CCR5) are obscured by the variable loops. The CD4-binding site lies in a deep pocket partly covered by the variable loops V1 and V2 (Kwong *et al.*, 1998; Wyatt *et al.*, 1998). The coreceptor-binding site is exposed only transiently following the conformational change that occurs after binding CD4 (Wyatt *et al.*, 1995). Consequently, the receptor-binding sites are poor in both immunogenicity and antigenicity.

¹ To whom correspondence and reprint requests should be addressed at Case Western Reserve University School of Medicine, Department of Medicine, Division of Infectious Disease, 10900 Euclid Avenue, Cleveland, OH 44106-4984. Fax: 216-844-1409. E-mail: mcho@po.cwru.edu.

The crystal structure of the core domain of gp120, along with the two amino-terminal domains of CD4 and Fab fragment of a monoclonal antibody 17b that binds CD4-induced domain, has been solved (Kwong *et al.*, 1998). Despite the fact that the crystallized protein has multiple missing segments of gp120 (viz. 52 and 19 amino acids at the amino- and carboxyl-terminus, respectively, and the variable loops V1/V2 and V3), the overall structural information from the crystal largely agree with biochemical, immunological, and genetic data available from other studies (Helseth *et al.*, 1991; Leonard *et al.*, 1990; Moore and Sodroski, 1996; Olshesky *et al.*, 1990). The poor immunogenic property of gp120 in eliciting broadly reactive Nabs against the virus is readily apparent from the analyses of the crystal structure of gp120 core. Based on the three-dimensional model derived from the crystal structure, a significant fraction of the exposed surface of the trimeric envelope glycoprotein on the virion is occupied by carbohydrate residues and the hypervariable regions (Wyatt *et al.*, 1998). Thus, to elicit Nabs that have broad cross-reactivity against a large number of genetically diverse HIV-1 isolates, a vaccine candidate has to be designed so that the conserved regions of gp120 are exposed as much as possible.

A number of strategies have been proposed or are being explored to elicit broadly reactive Nabs, including the use of fusion intermediates (LaCasse *et al.*, 1999), CD4-independent envelope glycoproteins (Hoffman *et al.*, 1999), glycosylation site mutants (Quinones-Kochs *et al.*, 2002; Reitter *et al.*, 1998), and variable loop-deleted envelope proteins (Barnett *et al.*, 2001; Johnson *et al.*, 2002; Lu *et al.*, 1998; Wyatt *et al.*, 1993). The rationale behind these approaches is to expose conserved epitopes of the protein (viz. CD4- or coreceptor-binding sites) that should be common to many HIV-1 isolates by either eliminating shields (i.e., carbohydrates or variable loops) or by capturing various transitional states of the envelope. In this study, the feasibility of using variable loop-deleted envelope glycoproteins as vaccine candidates against HIV-1 was evaluated. The specific goals of the study are (1) to identify immunogenic epitopes in the wild-type envelope glycoprotein of a dual tropic HIV-1 isolate (DH12); (2) to determine whether it is possible to redirect antibody responses from variable to conserved regions of the protein by removing the variable loops; and (3) to compare the potency and breadth of neutralizing activity of antisera elicited by the wild-type and mutant envelope constructs.

RESULTS

Expression of HIV-1 envelope glycoprotein

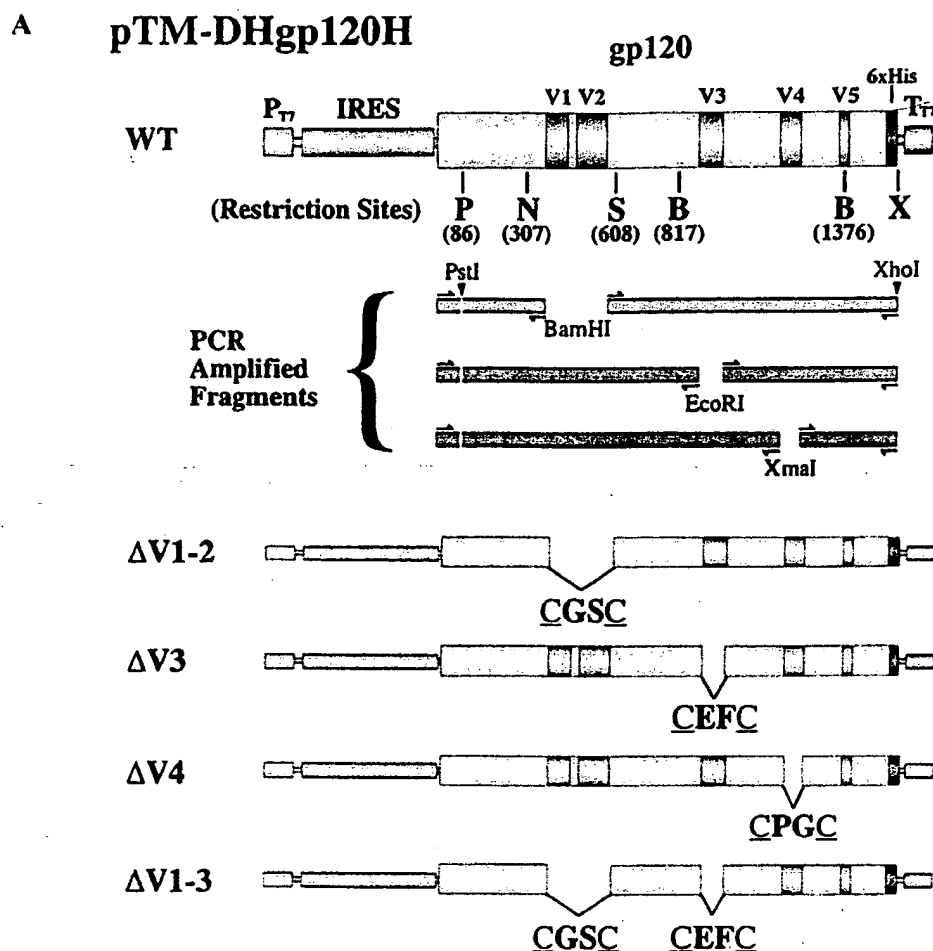
To characterize humoral immune response elicited by variable loop-deleted HIV-1 envelope glycoproteins, recombinant vaccinia viruses expressing gp160 and gp120

were generated. The recombinant viruses expressing the wild-type proteins have previously been described (Cho *et al.*, 2000, 2001; Lee *et al.*, 2000) and the variable loops V1-2, V3, V4, and V1-3 were deleted, as illustrated in Fig. 1. The viruses encoding gp160 express the protein under the control of vaccinia virus immediate-early promoter P7.5. In contrast, transcription of gp120 is controlled by T7 promoter. Thus, gp120 is expressed when cells are coinfecting with gp120-encoding viruses and another recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) (Fuerst *et al.*, 1986). Gp120 is tagged with six histidine residues at the carboxyl-terminus to facilitate protein purification. During the variable loop deletion procedure, restriction sites *Bam*HI, *Eco*RI, and *Xba*I were introduced at the V1-2, V3, and V4 loops, respectively. As a result, the variable loops were replaced with dipeptides Glycine-Serine, Glutamic acid-Phenylalanine, and Proline-Glycine, respectively.

Expression of envelope glycoproteins by the recombinant vaccinia viruses is shown in Fig. 2. All of the mutant gp120s were expressed and secreted into the culture medium as efficiently as the wild-type gp120 (Fig. 2A). As expected, Δ V1-3 gp120 exhibited the greatest increase in mobility, followed by Δ V1-2. Only a slight increase in mobility was observed for Δ V3 and Δ V4. All gp120s bound Ni-NTA resin, although mutant proteins exhibited somewhat weaker affinity than the wild-type. As a result, lower salt concentration was used during the purification procedure. All of the gp160s were expressed efficiently (Fig. 2B). The variable loop-deleted gp160s exhibited similar changes in mobility as for corresponding gp120s. Wild-type gp160 was processed efficiently as indicated by the large amount of gp120 in culture medium. Among the variable loop-deleted mutant proteins, only Δ V1-2 was processed efficiently. While some processing was observed for Δ V3 and Δ V4, no processing was observed for Δ V1-3. These results suggest that intact variable loops V3 and V4 are important for maintaining correct conformation of the protein necessary for protein processing. Not unexpectedly, none of the mutant gp120 clones, including the Δ V1-2 construct, exhibited any detectable fusion activity (data not shown) using the highly sensitive cell-to-cell fusion assay we previously described (Lee *et al.*, 1999).

Immunization and evaluation of antibody response

We have previously demonstrated that immunization of macaques with recombinant vaccinia virus expressing gp160, followed by boosting with purified recombinant gp120, could elicit potent neutralizing antibodies that protect vaccinated animals (Cho *et al.*, 2001). A similar prime-boost vaccine strategy was used (Table 1). Mice were immunized with either the wild-type or the variable loop-deleted envelope glycoprotein. The animals were initially primed with recombinant vaccinia viruses ex-



B Primers

F-env	5' -ACAGTGCATATGAGAGTGATGGGGATCAGG-3'	
R-env	5' -GGGCCCCCTCGAGTTAATGGTGATGATGGTGATGCTTTTTTCTCTCTGCACCACTC-3'	
ΔV1-2 (U)	5' -ATGTAAGGATCCACAGAGTGGGGTTAATTTTAC-3'	(355-375)
(D)	5' -AGGTTGGGATCCTGTAAACACCTCAACCCCTTAC-3'	(586-605)
ΔV3 (U)	5' -GTTGGGGAATTCACAATTAATTTCTACAG-3'	(872-888)
(D)	5' -AGAAAAGAATTCGTAAACATTAGTAAAGTAAATGG-3'	(988-1011)
ΔV4 (U)	5' -TTTTTCCCGGACAGTAGAAAAATCCCTCC-3'	(1129-1149)
(D)	5' -ATCACACCCGGTGCAGATAAAACAAATTATAAAC-3'	(1234-1257)

FIG. 1. Construction of variable loop-deleted HIV-1 envelope glycoprotein. (A) Schematic diagram of wild-type gp120 expression vector, pTM-DHgp120H (Lee *et al.*, 2000). The transcription of HIV-1_{DH12} gp120 is under the control of T7 promoter (P_{T7}) and T7 terminator (T_{T7}). The translation of the protein is enhanced by the presence of internal ribosome entry site (IRES). The protein is tagged with six histidine residues (6xHis) at the carboxyl-terminus to facilitate protein purification. The positions of endonuclease restriction sites *Pst*I (P), *Nsi*I (N), *Sma*I (S), *Bgl*II (B), and *Xho*I (X) are indicated. PCR-amplified fragments used to generate mutant constructs and the relative positions of the primers used for the PCR reaction are shown. During the cloning procedure, restriction sequences for *Bam*HI, *Eco*RI, and *Xba*I were introduced into the V1-2, V3, and V4 loops, resulting in insertion of dipeptides GS, EF, and PG, respectively, between the cysteine residues at the base of the variable loops. (B) Primers used for the PCR reaction. F- and R-env refer to forward and reverse primers at the 5' and 3' of gp120 gene, respectively. The coding sequences are indicated by bold phase, the *Xho*I site is underlined, and six histidine residues are boxed in. The internal primers used to amplify either upstream (U) or downstream (D) PCR fragments (relative to each variable loop) for generating ΔV1-2, ΔV3, and ΔV4 gp120s are indicated. The restriction sites introduced into the primers are underlined and the coding regions (up to the cysteine residues) are indicated by bold phase. The nucleotide numbering is based on HIV-1_{DH12} strain.

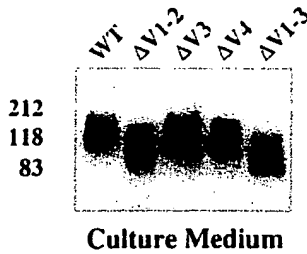
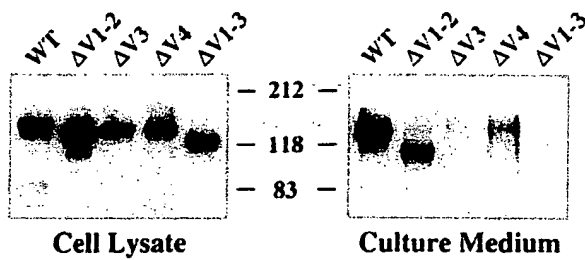
A gp120**B gp160**

FIG. 2. Expression of the wild-type and variable loop-deleted gp120s (A) and gp160s (B). Gp120 was expressed in HeLa cells by coinfection with recombinant vaccinia viruses that encode various gp120s under the control of T7 promoter and a second virus that expresses T7 RNA polymerase (vTF7-3). Because these constructs lack gp41 region, gp120 is secreted directly into cell-culture medium. In contrast, gp160 was expressed by infection with a single recombinant vaccinia virus that encodes gp160 under the control of vaccinia P7.5 promoter. While gp160 is found only in cell lysate (i.e., membrane-associated), most of the processed gp120 is shed into culture medium. Envelope proteins were detected by Western immunoblot using polyclonal rabbit anti-gp160 antiserum.

pressing gp160. For second and third immunizations on 4 and 11 weeks post-initial immunization, respectively, mice were immunized with both the recombinant vaccinia viruses and the purified recombinant gp120s. For the final boost on Week 17, mice were immunized with

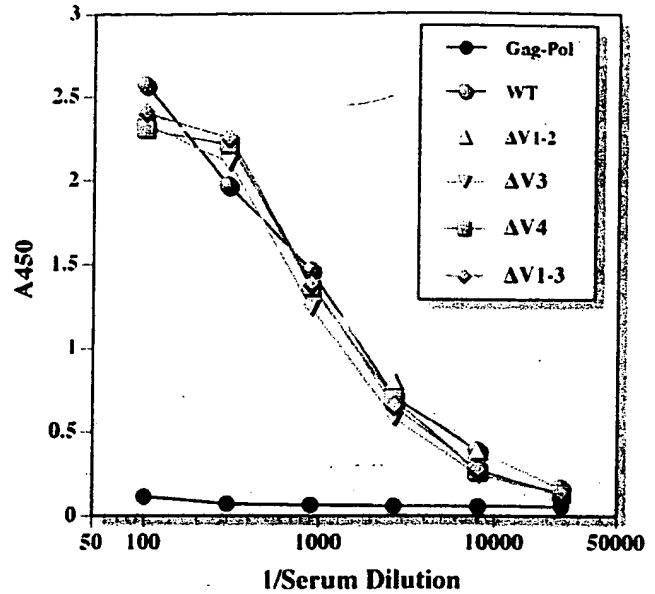


FIG. 3. Antibody response against gp120. Antisera from animals immunized with either the wild-type or the variable loop-deleted mutant envelope proteins were analyzed by ELISA. Wild-type gp120 from HIV-1_{DH12} was used as antigen. The absorbance (at 450 nm) is shown as a function of serum dilution factor. Antisera from animals immunized with SIV gag-pol was used as a negative control. The final bleed, obtained after the last boost, was used.

gp120 only. As a negative control, one group of mice was immunized with a recombinant vaccinia virus expressing SIV gag-pol on weeks 0, 4, and 11, and with recombinant p55 protein on Week 17. Expression of SIV gag-pol and purification of p55 will be described in detail elsewhere (Y. B. Kim, D. P. Han, and M. W. Cho, unpublished data).

Humoral immune response against HIV-1 envelope glycoprotein was evaluated by enzyme-linked immunosorbent assay (ELISA). An assay performed with sera collected after the final immunization is shown in Fig. 3.

TABLE 1
Vaccine Design and Immunization Schedule

Vaccine	No. of mice	1 (Week 0)	2 (Week 4)	3 (Week 11)	4* (Week 17)
SIVgagpol	4	wSIVgagpol	wSIVgagpol	wSIVgagpol	SIV p55
W.T.	4	wDHenv	wDHenv gp120	wDHenv gp120	gp120
ΔV1-2	4	wDHenvΔV1-2	wDHenvΔV1-2 gp120ΔV1-2	wDHenvΔV1-2 gp120ΔV1-2	gp120ΔV1-2
ΔV3	4	wDHenvΔV3	wDHenvΔV3 gp120ΔV3	wDHenvΔV3 gp120ΔV3	gp120ΔV3
ΔV4	4 ^b	wDHenvΔV4	wDHenvΔV4 gp120ΔV4	wDHenvΔV4 gp120ΔV4	gp120ΔV4
ΔV1-3	4	wDHenvΔV1-3	wDHenvΔV1-3 gp120ΔV1-3	wDHenvΔV1-3 gp120ΔV1-3	gp120ΔV1-3

Note. Recombinant proteins are indicated in bold.

* Administered with 50 μg of QS-21 as adjuvant.

^b One animal died during relocation to a new facility.

TABLE 2
Neutralization of HIV-1 gp140 Pseudoviruses With Immunized Mice Sera*

Vaccine	DH12	Bal	AD8	89.6	RF
SIVgag-pol	—	—	—	—	—
W.T.	1:16	—	—	—	—
Δ V1-2	1:4	—	—	—	—
Δ V3	—	—	—	—	—
Δ V4	1:8	—	—	—	—
Δ V1-3	—	—	—	—	—
IgG b12 ^b	25	12.5	12.5	12.5	0.78

* Serum dilution required to yield 90% reduction of 50 IFU of gp140-pseudotyped virus.

^b Antibody concentration (μ g/ml) that yielded complete neutralization of the pseudoviruses.

—, No neutralizing activity was detected at 1:4 dilution.

The antibody response against gp120 was about the same for all envelope-immunized vaccine groups with endpoint titers of approximately 1:30,000. This represents approximately 10-fold higher titer compared to that found in HIV-1-infected patients when measured under the same assay condition. Envelope-specific antibody was not detected in preimmune sera (data not shown) or in animals immunized with SIV gag-pol (Fig. 3). Antibody titers of sera collected after the third immunization were similar to those collected after the final immunization for all vaccine groups except for those immunized with Δ V3 and Δ V1-3, which were about threefold lower (data not shown). This result suggests that V3- and V1-3-deleted envelope glycoproteins might be slightly less immunogenic than the other envelope constructs. The animals were not bled after the first and second immunization because we normally do not detect or detect only low levels of antigen-specific antibody response. Mice immunized with recombinant vaccinia virus expressing SIV gag-pol and p55 protein mounted a strong antibody response against SIV p55 as well as p27 and p17 (data not shown).

Neutralization activity

To determine whether the variable loop-deleted envelope constructs could elicit Nabs that are more potent and/or have greater breadth in reactivity than those elicited by the wild-type, neutralizing activity of sera collected after the final immunization was evaluated. We have recently described a rapid, highly sensitive neutralization assay that uses β -galactosidase-encoding murine leukemia virus (MuLV) pseudotyped with HIV-1 gp140 (Kim *et al.*, 2001). Neutralizing activity of antisera against MuLV pseudotyped with gp140 of HIV-1 isolates DH12, Bal, AD8, 89.6, and RF is shown in Table 2. Against DH12, which is the HIV-1 strain used to generate gp160 and gp120 immunogens, the group immunized with the

wild-type protein exhibited the highest neutralizing activity (1:16), followed by those immunized with Δ V4 (1:8) and Δ V1-2 (1:4). No neutralizing activity was detected in animals immunized with Δ V3, Δ V1-3, or SIV gag-pol. None of the vaccine groups had any detectable neutralizing activity against other HIV-1 isolates, including the group immunized with wild-type protein. The inability to neutralize these viruses is not due to the inherently resistant nature of pseudoviruses since they were neutralized by broadly neutralizing monoclonal antibody IgG b12 (Table 2). No neutralizing activity was detected in prebleeds from any of the animals.

Characterization of immunogenic epitopes

Although the variable loop-deleted envelope glycoproteins we have generated were not able to elicit neutralizing antibodies that are either potent or broadly reactive, understanding immunogenic properties of these antigens in comparison to those of the wild-type protein could facilitate design of a better immunogen in the future. To identify epitopes that are immunogenic, ELISA was performed against 93 gp120 peptides that are 15 amino acids in length and overlap by 10 amino acids (Table 3). These peptides cover the entire length of HIV-1_{DH12} gp120, except for the signal peptide. The antibody response against these peptides is shown in Fig. 4. In animals immunized with the wild-type envelope glycoprotein, 16 highly immunogenic epitopes (arbitrarily defined as having A_{450} of greater than 1.0) were identified (Fig. 4, top panel, and Table 3). These epitopes were primarily located in the C1, V1/V2, and C5 regions of gp120, which accounted for 75% of all immunogenic epitopes. The immunogenic epitope identified in the V3 loop (peptide 56) contained the Gly-Pro-Gly-Arg sequence situated in the middle of the peptide. These amino acids, located at the tip of the V3 loop, have been previously shown to be important in eliciting isolate-specific Nabs (Goudsmit *et al.*, 1988; Javaherian *et al.*, 1990; McKeating *et al.*, 1989; Palker *et al.*, 1988; Rusche *et al.*, 1988). Although the adjacent peptides (nos. 55 and 57) also contained GPGR motif, they were not immunoreactive, suggesting the importance of proper geometry of the motif in order for it to be antigenic. There are two cautionary notes regarding the immunogenic epitopes identified in this study. First, the epitopes we identified represent only those that are linear and those that can be folded into by 15 amino acid long peptides. Epitopes that are discontinuous or those that require longer peptides would not be identified. Second, since the peptides we used overlap by 10 amino acids, we do not know whether multiple immunoreactive peptides that are adjacent to each other (i.e., peptides 12/13, 22/23/24, and 87/88) represent multiple independent epitopes or a single epitope that can be displayed by different peptides.

Antisera from the animals immunized with the variable

TABLE 3

List of Peptides Used for ELISA

Number	Sequence	Number	Sequence	Number	Sequence
1	AEQLVVTYYGVPVW	32	DRNITSYRLISCNTS	63	TLKRVVEKLREKFEN
2	VTVYGGVPVWKEANT	33	SYRLISCNTSTLTQA	64	VEKLREKFENKTIVF
3	GVPVWKEANTTLFCA	34	SCNTSTLTQACPVS	65	EKFENKTIVFNKSSG
4	KEANTTLFCASDAKA	35	TLTQACPVSFEPIP	66	KTIVFNKSSGGDPEI
5	TLFCASDAKAYDEV	36	CPKVSFEPIPIHYCA	67	NKSSGGDPEIVMHSF
6	SDAKAYDEVHNVWA	37	FEPIPIHYCAPAGFA	68	GDPEIVMHSFNCGGE
7	YDEVHNVWATHACV	38	IHYCAPAGFAILKCK	69	VMHSFNCGGEFFYCN
8	HNVWATHACVPTDPN	39	PAGFAILKCKDKKFN	70	NCGGEFFYCNKKLF
9	THACVPTDPNPQEI	40	ILKCKDKKFNGTGPG	71	FFYCNKKLFNSTWN
10	PTDPNPQEIILENV	41	DKKFNGTGPCTNVST	72	TKKLFNSTWNGTEGS
11	PQEIILENVTEDFNM	42	GTGPCTNVSTVQCTH	73	NSTWNGTEGSYNIEG
12	LENVTEDFNMWKNM	43	TNVSTVQCTHGIRPV	74	GTEGSYNIEGNDTIT
13	EDFMWKNMVEQMH	44	VQCTHGIRPVSTQL	75	YNIEGNDTITLPCRI
14	WKNMVEQMHEDIIS	45	GIRPVSTQLLNGS	76	NDTITLPCRIKOIIN
15	VEQMHEDIISLWQDS	46	VSTQLLNGSLAEEL	77	LPCRIKOIINMWQEV
16	EDIISLWQDSLKPCV	47	LLNGSLAEELVIRS	78	KOIINMWQEVGKAMY
17	LWQDSLKPCVKLTPL	48	LAEEELVIRSSNFTD	79	MWQEVGKAMYAPPIS
18	LKPCVKLTPLCVTLH	49	VVIRSSNFTDNAKII	80	GKAMYAPPISGQIWC
19	KLTPLCVTLHCTDLK	50	SNFTDNAKIIIVQLN	81	APPISGQIWCSSNIT
20	CVTLHCTDLKNGTNL	51	NAKIIIVQLNETVEI	82	GQIWCSSNITGLLLT
21	CTDLKNGTNLKNGTK	52	IVQLNETVEINCTRP	83	SSNITGLLLTRDGGK
22	NGTNLKNGTIIGKS	53	ETVEINCTRPNNNTR	84	GLLLTRDGGKNSSTE
23	KNGTKIIGKSMRGEI	54	NCTRPNNNTRKGITL	85	RDGGKNSSTEIFRPG
24	IIGKSMRGEIKNCSF	55	NNNTRKGITLGPGRV	86	NSSTEIFRPGGGDMR
25	MRGEIKNCSFNVTKN	56	KGITLGPGRVFYTTG	87	IFRPGGGDMRDNRWS
26	KNCSFNVTKNIIDKV	57	GPGRVFYTTGEIVGD	88	GGDMRDNRWSELYKY
27	NVTKNIIDKVKEYA	58	FYTTGEIVGDIRKAH	89	DNWRSELYKYKVVVR
28	IIDKVKEYALFYRH	59	EIVGDIRKAHCNISK	90	ELYKYKVVVRVEPLGI
29	KKEYALFYRHVVPI	60	IRKAHCNISKVKWHN	91	KVVVRVEPLGIAPTAK
30	LFYRHVVPIIDRNIT	61	CNISKVKWHNTLKR	92	EPLGIAPTAKARRRV
31	DVVPIDRNITSYRLI	62	VKWHNTLKRVEKL	93	APTAKARRVQREKR

Note. Immunoreactive peptides are boxed (see Fig. 4). Peptides that include the variable regions are shaded: V1/V2: 19–33; V3: 53–60; V4: 70–76; V5: 84–86.

loop-deleted envelopes were also examined. The results are shown as A_{450} of wild-type subtracted from A_{450} of mutants for each peptide (Fig. 4, bottom four panels). Thus, the positive values represent greater immunogenicity for the mutant envelopes, while the negative values represent greater immunogenicity for the wild-type protein. Values close to zero represent little or no difference between the wild-type and mutant proteins. As predicted, sera from the animals immunized with $\Delta V1-2$ envelope lost immunoreactivity against the V1–2 peptides. While most peptides had similar immunoreactivity, six peptides were significantly more immunogenic (arbitrarily defined as the difference of greater than A_{450} of 1) compared to the wild-type (peptides 47, 54, 55, 85, 86, and 92). Peptides 54 and 55 are located in the V3 loop, while peptides 85 and 86 are located in the V5 region. Only two peptides were from the conserved regions of gp120 (C2 and C5).

Also as expected, antisera from the animals immunized with the $\Delta V3$ envelope lost immunoreactivity against peptide 56. Surprisingly, immunoreactivity

against the V1–2 loop peptides was also reduced almost as much as the $\Delta V1-2$ immunized group. This result suggests that conformation of the V3 loop has a significant influence on how the V1–2 loops are folded. Immunoreactivity against peptide 40 in the C2 region was also lost. In contrast, immunoreactivity against three peptides in C1 and C2 increased (7, 47, and 48). It is interesting that immunoreactivity against peptide 47 was enhanced by the removal of either V1–2 or V3 loops. Deletion of V4 loop reduced immunogenicity of peptides 6, 22, 30, and 61. In contrast, immunogenicity of peptides 11, 28, and to a lesser degree peptides 10, 92, and 93, was increased. The immunoreactivity pattern for the $\Delta V1-3$ group largely resembled that for the $\Delta V1-2$ and $\Delta V3$ groups combined (i.e., loss of reactivity against both V1–2 and V3 peptides). In addition, reactivity toward peptide 87 was lost. In contrast, reactivity toward several peptides in the C1 and C5 increased (peptides 11, 14, and to a lesser degree 10, 92, and 93).

To visualize immunogenic epitopes in a three-dimen-

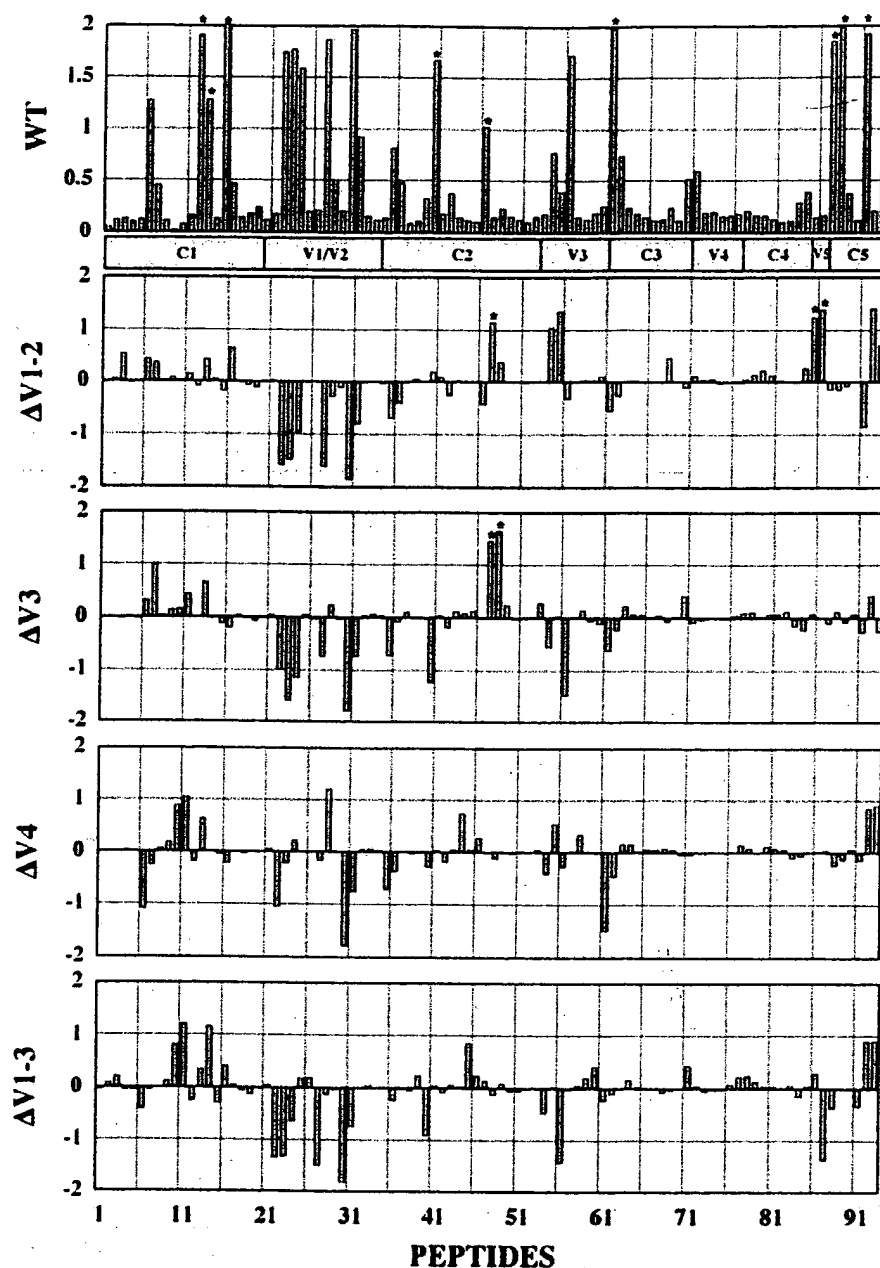


FIG. 4. Characterization of immunogenic epitopes by peptide ELISA. The final bleed was analyzed by ELISA against the peptides listed in Table 3. The peptides are labeled on the bottom of the graph (1–93). On the top, the absorbance (at 450 nm) for the antisera from the animals immunized with the wild-type envelope is shown for each peptide. For the variable loop-deleted envelopes (bottom four panels), the results are shown as A_{450} of wild-type subtracted from A_{450} of mutants for each peptide. Approximate locations of the conserved (C1–5) and variable (V1–5) regions are indicated.

sional model of gp120, we plotted the immunoreactive peptides onto the corresponding coordinates of gp120 crystal structure solved by Kwong *et al.* (1998) (Fig. 5). The peptides that reacted strongly to antisera from the animals immunized with the wild-type envelope glycoprotein are shown in Fig. 5A. Because the crystallized gp120 core has multiple missing segments of gp120 (*viz.* 52 and 19 amino acids at the amino- and carboxyl-terminus, respectively, and the variable loops V1/V2 and V3), not all immunogenic epitopes could be displayed on

the crystal structure. Only those indicated by asterisks in Fig. 4 are shown. By-and-large, most of the immunogenic peptides mapped to the inner domain of gp120 (peptides 12, 13, and 15 in red; peptide 40 in blue; peptides 87 and 88 in cyan; and peptide 91 in magenta), which has been referred to as "nonneutralizing face" (Wyatt *et al.*, 1998). Only peptides 46 in C2 (yellow) and 61 in C3 (green) (not including the immunogenic peptides in the variable loops) were situated in the outer domain of the protein. No peptides mapped to regions that are highly glycosy-

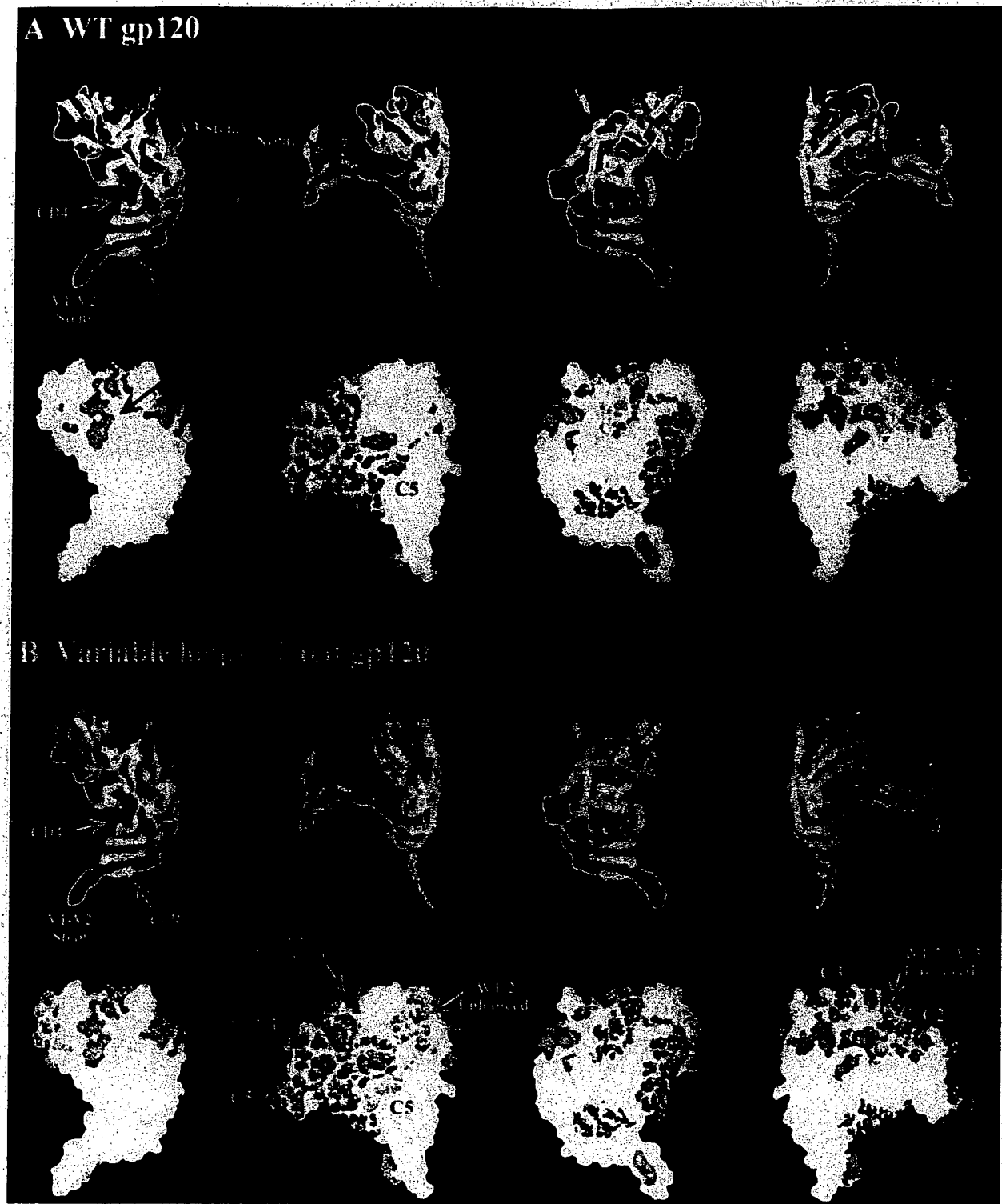


FIG. 5. Three-dimensional visualization of immunogenic epitopes. Immunoreactive peptides identified in Fig. 4 (marked by asterisks) were visualized by plotting them onto the corresponding segments of the known crystal structure of HXBc2 gp120 core (Kwong *et al.*, 1998). The peptides that reacted to antisera from animals immunized with the wild-type envelope are shown in (A). The upper portion of the panel shows ribbon diagram, while the lower portion shows the surface map of gp120. The left-most images depict the view from the target cell membrane. The successive images from the left to right show approximately 90° turn along the top-bottom axis. Thus, the third image from the left depicts the view from the orientation of the viral membrane. The receptor-binding sites (CD4 and coreceptor) and the stems of the missing variable loops V1/V2 and V3 are indicated. Also indicated are the amino- and carboxyl-termini of the gp120 core protein used to determine the crystal structure, and the residual carbohydrate moieties (*N*-acetyl-D-glucosamine and fucose, shown in blue-green). The immunoreactive peptides are color coded as the following: peptides 12, 13, and 15 (C1 region) in red; peptides 40 and 46 (C2 region) in blue and yellow, respectively; peptide 61 (C3 region) in green; and peptides 87/88 and 91 (C5 region) in cyan and magenta, respectively. Immunoreactive peptides to antisera from animals immunized with either V1/V2 or V3 are shown in (B). Peptides that exhibited enhanced reactivity by the deletion of V1-2 (peptides 85 and 86) are shown in gold. Peptides that were enhanced by the deletion of the V3 loop (peptide 48) or by either V1-2 or V3 loops (peptide 47) are shown in purple.

lated ("immunosilent face") or to areas where CD4 and coreceptors bind ("neutralizing face"). Interestingly, many of the immunoreactive peptides we identified overlapped sequences that form alpha helix. Whether this is due to possibilities that alpha helices are inherently immunogenic, or that they are easily detected by peptide ELISA, or both, is not known.

Peptides that are immunogenic in $\Delta V1-2$ and $\Delta V3$ envelopes are shown in Fig. 5B. Peptides that exhibited enhanced reactivity by $\Delta V1-2$ (peptides 85 and 86) were positioned in the junction between the inner and the outer domains (shown in gold). The peptides that were enhanced by $\Delta V3$ (peptide 48), or by $\Delta V1-2$ and $\Delta V3$ (peptide 47), are located in the outer domain (in purple). None of these peptides, however, mapped to the neutralizing face of the protein.

DISCUSSION

In this study, we examined immunological properties of wild-type and variable loop-deleted gp120s with an ultimate goal of identifying an immunogen that can elicit broadly reactive Nabs against HIV-1. The rationale behind this vaccine strategy is based on an observation that the removal of variable loops V1/V2 and V3 increases affinity of Nabs directed against the CD4-binding domain, presumably due to enhanced exposure of the epitope (Wyatt *et al.*, 1993). Additionally, variable loops V1/V2 (V2 in particular) are thought to mask the coreceptor-binding site, which is transiently exposed only after gp120 binds CD4 (Cao *et al.*, 1997; Wyatt *et al.*, 1995). Disappointingly, the variable loop-deletion mutant envelope constructs we generated did not elicit either more potent or broadly cross-reactive Nab responses compared to the wild-type envelope.

Immunogenicity of variable loop-deleted envelopes has been evaluated in two other studies (Barnett *et al.*, 2001; Lu *et al.*, 1998). In the study by Lu *et al.* (1998), immunogenicity of three different wild-type and V1/V2/V3-deleted envelope constructs (gp120, gp140, and gp160) was compared in rabbits using DNA vaccine approach. Although the variable loop-deleted antigens were better than the wild-type in eliciting antibodies that bind mutant gp120, the antibodies elicited by the wild-type antigens were better in binding the wild-type gp120. More importantly, only the wild-type envelopes (gp120 and gp140) were able to elicit Nabs. These results suggested that while the removal of the variable loops generated or exposed new immunogenic determinants, they are not present or accessible in the wild-type protein. Consequently, antibodies to these determinants did not neutralize the virus. Barnett *et al.* (2001) examined immunogenicity of HIV-1_{SF162}-derived gp140 construct with deletion of the V2 loop only (30 amino acids), in rabbits (DNA) and in rhesus macaques (DNA prime-protein boost). They have demonstrated that V2 loop-deleted

immunogen, compared to the wild-type envelope, elicited similar (rabbit) or lower (macaque) ELISA antibody titer against gp140. However, the mutant envelope elicited slightly higher Nab titer against the wild-type virus and modestly broader, albeit weak, neutralizing activity against a limited number of heterologous primary HIV-1 isolates. Despite some success, the authors of the study concluded that additional modifications must be introduced to elicit Nabs with increased potency and breadth.

The moderate success reported by Barnett *et al.* (2001), in contrast to our study, and that of Lu *et al.* (1998), might be attributed to the use of a mutant envelope construct that contains much smaller deletion, which does not abrogate infectivity of the virus containing the mutation. Although our V3- and V4-loop deletions were as small as their V2 loop deletion (33 and 28 amino acids, respectively), deletions of the V3 and V4 loops might be more deleterious than that of the V2 loop. That is, both V3- and V4-loop deleted gp160 constructs exhibited a significant defect in gp160 processing, whereas processing of the V1/V2-deleted construct was largely unaffected (Fig. 2). Furthermore, while viable HIV-1 and SIV variants with V1/V2 or V2 loop deletions have been isolated (Cao *et al.*, 1997; Johnson *et al.*, 2002; Stamatos and Cheng-Mayer, 1998), viable virus containing either V3 or V4 loop deletions, to our knowledge, has never been observed. In this regard, the use of envelope constructs with less drastic deletion of the variable loops might result in a better outcome. In our study, we replaced variable loops V1/V2, V3, and V4 with dipeptides (GS, EF, and PG, respectively). In the study reported by Lu *et al.* (1998), trimeric peptide (GAG) replaced the V1/V2 and V3 loops. One of the remaining challenges for successfully using variable loop-deleted envelopes as immunogens might be generating deletion mutants that maintain functionality of the envelope.

To gain insights as to why the variable loop-deleted envelope constructs failed to elicit enhanced Nab response, immunogenic epitope analysis was performed using peptide ELISA (Figs. 4 and 5). For HIV-1_{DH12}, a dual tropic clade B primary isolate that can utilize either CXCR4 or CCR5 (X4R5) (Cho *et al.*, 1998), the single most immunogenic determinant was the variable loops V1/V2. This is consistent with our previous observation that immune serum from a chimpanzee infected with HIV-1_{DH12} could efficiently immunoprecipitate DH12 gp120 and a chimeric gp120 that contained the DH12 V1/V2 region in the background of HIV-1_{AD8} envelope; AD8 gp120 or chimeric gp120s that contained the V3, V4, or V5 regions of DH12 were not immunoprecipitated efficiently (Cho *et al.*, 2000). The V1/V2 region, together with the conserved regions C1 and C5, accounted for 75% (12 of 16) of all immunoreactive peptides identified in gp120. Other immunogenic peptides included two in the C2 region, one in the V3 loop, and one in the junction between V3 and C3. In contrast, much of the C3, V4, C4,

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Influence of N-Linked Glycans in V4-V5 Region of Human Immunodeficiency Virus Type 1 Glycoprotein gp160 on Induction of a Virus-Neutralizing Humoral Response

Anders Bolmstedt, *Sigrid Sjölander, †John-Erik S. Hansen, *Lennart Åkerblom, Anna Hemming, ‡Shiu-Lok Hu, *Bror Morein, and Sigvard Olofsson

Department of Clinical Virology, University of Göteborg, Göteborg; *Department of Veterinary Virology, Biomedical Center, Uppsala Sweden; †Laboratory for Infectious Diseases, Hvidovre Hospital, Hvidovre, Denmark; and ‡Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington, U.S.A.

Summary: One of the functions of N-linked glycans of viral glycoproteins is protecting otherwise accessible neutralization epitopes of the viral envelope from neutralizing antibodies. The aim of the present study was to explore the possibility to obtain a more broadly neutralizing immune response by immunizing guinea pigs with gp160 depleted of three N-linked glycans in the CD4-binding domain by site-directed mutagenesis. **Mutant and wild type gp160** were formulated into immunostimulating complexes and injected s.c. into guinea pigs. Both preparations induced high serum antibody response to native gp120 and V3 peptides. **Both preparations also induced antibodies that bound equally well to the V3 loop or the CD4-binding region, as determined by a competitive enzyme-linked immunosorbent assay (ELISA).** The sera from animals, immunized with mutated glycoprotein, did not neutralize nonrelated HIV strains better than did sera from animals, immunized with wild type glycoprotein. **Instead, a pattern of preferred homologous neutralization was observed, i.e., sera from animals immunized with mutant gp160, neutralized mutant virus better than wild type virus, and vice versa.** These data indicated that elimination of the three N-linked glycans from gp160 resulted in an altered local antigenic conformation but did not uncover hidden neutralization epitopes, broadening the immune response. **Key Words:** Immunogen—Oligosaccharide—HIV-1—gp120—N-linkage.

The envelope glycoprotein gp120 from HIV-1 is equipped with >20 N-linked glycans (1,2), which by virtue of their large molecular volume must cover most of the peptide surface of the glycoprotein (3,4). These oligosaccharides are essential for several functions necessary for HIV to infect its target cell (5). In addition, N-linked glycans are important

in the interplay between a virus and the immune response of an infected host (6,7). In this context, we may discern two activities: the ability of carbohydrates of gp120 to structurally maintain an appropriate antigenic conformation and their capacity to shield potential neutralization epitopes from antibodies (8-14). This latter activity is possible because most of the structural information of viral N-linked glycans is derived from the host cell, and the oligosaccharides are therefore tolerated as self structures by the immune response [reviewed in (4)].

We have shown that it is possible to eliminate by

Address correspondence and reprint requests to S. Olofsson, Department of Clinical Virology, University of Göteborg, Guldhedsgatan 10B, S-413 46 Göteborg, Sweden. E-mail: sigvard.olofsson@microbio.gu.se

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site-directed mutagenesis as many as three N-linked glycans (associated with N406, N448, and N463) from the CD4-binding region of gp120 without interfering with its capacity to bind to CD4 or to induce subsequent fusion between adjacent membranes (11,15,16). Moreover, a mutated HIV clone, lacking the three N-linked glycans, is as infectious as its parent wild type strain (A. Hemming et al., unpublished observations). These data indicate that the three N-linked glycans are not essential for the virus in its infectious cycle. However, the positions of the three glycans are relatively conserved (17), suggesting that they still fill some function sufficiently important to merit their presence on the glycoprotein. Owing to the vicinity of these three N-linked glycans to the biologically important CD4-binding domain of gp120, one possible function could be shielding of this conserved and seemingly vulnerable area from neutralizing antibodies. Such carbohydrate-related protection could operate either by protecting the virus from circulating antibodies to the CD4-binding domain or by preventing the generation of antibodies to this region. This latter aspect is supported by data of Benjouad et al. (10) showing that desialylation of gp160 results in an immunogen with capacity to induce a more broadly reactive immune response. In the present report, we analyzed whether removal of defined N-linked oligosaccharide side chains within the CD4-binding domain of gp160-iscoms influenced the induction of a neutralizing humoral response against HIV-1. That could, for example, occur by broadening the virus neutralizing capacity to heterologous isolates or possibly in the opposite direction by focusing the response to the variant used for immunization.

MATERIAL AND METHODS

Cell Cultures

BSC-40 cells (ATCC#CCL 2b:BSC-1) were propagated in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 10,000 IU/ml penicillin and 100 µg/ml streptomycin. The CD4⁺ T-cell lines MT-4 and H9 were propagated in RPMI₁₆₄₀ with 10% heat-inactivated fetal calf serum, 2 µM glutamine, 10,000 IU/ml penicillin and streptomycin, and 20 µg/ml gentamicin. All cells were cultured at 37°C and 5% CO₂.

Construction of Recombinant Vaccinia Virus Expressing Mutated HIV-1-Specified gp160/gp120

Recombinant vaccinia viruses expressing the wild type gp160 sequence or a mutated gp160 sequence, gp160_{A123}, lacking the N-linked glycans associated with N406, N448, and N463, were

constructed as previously described using published principles for site-directed mutagenesis and construction of recombinant vaccinia virus (15,16,18-20). Recombinant gp160 was expressed and purified according to Klaniecki et al. (20) and formulated in iscoms (21), prepared as described by (22).

Immunization of Guinea Pigs

Guinea pigs in groups of 10 were immunized s.c. in the neck with 20 µg of purified wild type gp160 or mutant gp160 (gp160_{A123}) formulated in iscoms (22) and boosted 6 weeks later with the same constructs (Table 1). The guinea pig sera tested were collected 6 weeks after the second immunization.

Construction and Expression of Infectious Clones of Mutated HIV-1

The proviral plasmid is based on pBRU-2 (23), but contains a BH 10 strain insert (11) where the mutations were created, as described previously (24; A. Hemming et al., unpublished observations). Infectious virus clones were obtained after transfection of H9 cells (Table 2). The virus containing wild type gp120 sequence was designated HIV-1_{BRU}, and the virus expressing gp120 lacking the three N-linked glycans was designated HIV-1_{BRU-A123}. The behavior in cell culture of HIV-1_{BRU-A123} is indistinguishable from that of HIV-1_{BRU} (A. Hemming et al., unpublished observations). HIV-1 variants analyzed in the present study are presented in Table 2.

Formation of gp160 Iscoms

Iscoms were prepared by the dialysis method (25) to contain the HIV-1_{BRU} or HIV-1_{BRU-A123} envelope gp160 (22). To 1 mg of gp160 in 1 ml of phosphate-buffered saline (PBS) containing 2% MEGA-10 was added 0.1 ml of a lipid solution consisting of 10 mg each of cholesterol and phosphatidyl choline, dissolved in 1 ml of a 20% MEGA-10/H₂O solution. Quil A (0.1 ml) was added from stock solution (100 mg/ml). The mixture was sonicated in a sonicator water bath, left at room temperature for 60 min, and dialyzed against PBS overnight at room temperature. This was followed by another dialysis at 4°C overnight. After dialysis, the sample was applied to a 10% sucrose cushion in PBS and centrifuged in a Kontron TST 41.14 for 18 h, 20°C, at 39,000 rpm. The pelleted material was dissolved in 1 ml PBS. The preparation was analyzed by electron microscopy for morphology, and the incorporated glycoprotein was analyzed by SDS-PAGE and Western blot to verify the inclusion of gp160 into iscoms (not shown). About 80% of the glycoprotein used for iscom formation was recovered in the iscom pellet.

Radioimmunoprecipitation Assay (RIPA)

BSC-40 cells were inoculated and radiolabeled as previously described (11). Recombinant vaccinia virus encoding the wild-type *env* sequence was designated v-J1(11K), and virus encoding gp160, lacking the N-linked glycans associated with N406, N448, and N463, was designated v-J7(11K). Labeled cell extracts and culture supernatants were mixed with pooled sera from guinea pigs immunized with wild type gp160 or gp160_{A123} and incubated for 1.5 h at 4°C. Heat-inactivated formalin-fixed *Staphylococcus*

TABLE 1. ELISA titers of sera from guinea pigs after immunization with wild type gp160 or gp160_{A123} formulated in iscoms

Animal group	Immunogen	Target antigen			
		gp160 ^a		V3-peptide ^b	
		Mean titer ^c	Range ^d	Mean titer ^c	Range ^d
I	gp160-iscoms	1,334,000	(977,000-3,125,000)	10,700	(6,700-21,700)
II	gp160 _{A123} -iscoms	641,600	(529,000-1,200,000)	9,600	(5,600-44,000)

ELISA, enzyme-linked immunosorbent assay.

^a ELISA titers of sera to gp160.

^b ELISA titers of sera to a synthetic peptide representing the tip of the V3_{HXB2}-loop (28).

^c All titers are expressed as geometric means.

^d 95% confidence (n = 10) intervals for the ELISA titers are shown within parentheses.

cells were then added and mixed for 1 h at 4°C. The samples were then washed twice in TBS containing 1% Triton X-100 and 0.1% bovine serum albumin, and twice in TBS prior to 9.25% SDS-PAGE and fluorography (Amersham).

Neutralization Assay

Neutralization assays were performed essentially as described by Hansen et al. (26). Briefly, in a total volume of 500 µl, 2×10^5 MT-4 cells in fresh growth medium were inoculated with 100 TCID₅₀ virus for 2 h at 37°C, 5% CO₂. Before inoculation, the virus inoculum was preincubated for 1 h at room temperature with heat-inactivated (56°C, 30 min) guinea pig serum. After inoculation, the cells were washed extensively, and quadruplicates of 0.4×10^5 cells were cultured in plain growth medium in 96-well cell culture plates. At day 4, the HIV antigen concentration in the supernatants was measured using an in-house double sandwich antigen capture enzyme-linked immunosorbent assay (ELISA) (26). All in vitro infection experiments were performed under identical conditions and included a culture control in which untreated HIV was used. Results were expressed as HIV antigen concentration relative to these untreated control cultures (percent), and neutralization was defined as an HIV-antigen concentration <20% of control cultures. Preimmunization sera all had neutralization titers <20.

Glycoprotein and Peptide ELISA

Microtiter plates (Nunc, Roskilde, Denmark) were coated with gp160 (1 µg/ml) or a synthetic peptide (NNTRKSIRIQGP-URAFVTIGKIG) (2 µg/ml) representing the V3-region of the HIV-2_{IIIIB} strain (kindly provided by Dr. C. Saxinger, National Cancer Institute, Bethesda, MD, U.S.A.) in 50 mM carbonate buffer (100 µl/well), pH 9.6, overnight at 4°C. The plates were

blocked in PBS containing 0.05% Tween-20 and 5% fat-free skim milk powder (blocking buffer) for 1 h at room temperature. After wash two times in PBS containing 0.05% Tween-20 (PBS-Tween), serum samples were added in threefold dilutions, starting from 1:50, in PBS containing 0.05% Tween-20 and 2% fat-free skim milk powder (PBS-Tween-milk), and the plates were incubated for 2 h at room temperature. The plates were washed three times in PBS-Tween and subsequently incubated for 1 h at room temperature with peroxidase conjugated rabbit anti-guinea pig immunoglobulins (P141, Dakopatts, Glostrup, Denmark) diluted 1:100 in PBS-Tween-milk. The plates were washed three times in PBS before addition of tetramethylbenzidine substrate solution (SVANOVA, Uppsala, Sweden) prepared according to the manufacturer's instructions, followed by incubation for 10 min at room temperature. The color reaction was stopped by addition of 2 M H₂SO₄, and the absorbance was monitored at 405 nm. Titer endpoints, calculated from linear regression of the linear part of the titration curve, were estimated as the dilution of serum giving an absorbance at the background level (i.e., reaction of prebleed sera) plus three SDs.

Competitive ELISA

Microtiter plates were coated with wild type or gp160_{A123} (1 µg/ml) and blocked as described earlier. Threefold dilutions of sera made in blocking buffer (50 µl/well) and starting from 1:50 were preincubated for 1 h at room temperature in a separate microtiter plate with biotinylated human monoclonal antibody GP13 directed to an epitope at least partly overlapping the CD4-binding domain of gp120 (27), or with biotinylated mouse monoclonal antibody F58/H3 directed to the V3-region (28) at a concentration of 2.5 or 1.25 µg/ml, respectively, in blocking buffer. The antibody mixture was transferred to the gp160-coated microtiter wells and incubated for an additional 2 h at room tem-

TABLE 2. Viral constructs used in neutralization assays

Virus clone	Properties	Locations of point mutations
HIV-1 _{BRU}	Based on pBRU2, (23) containing a BH-10 insert (11)	None
HIV-1 _{BRU-A123}	Prepared from HIV-1 _{BRU} . Lacks three glycosylation sites in CD4-binding domain (Hemming et al., unpublished observations)	TKGSS(N ₄₀₆ → Q)NTEGSD IRCSS(N ₄₄₈ → Q)ITGLL GGNSN(N ₄₆₃ → Q)ESESIF CTRP(N ₅₀₈ → Q)NTRKS
HIV-1 _{BRU-N308}	Based on pBRU2, lacking one glycosylation site in the V3-loop (24,31)	None
HIV-1 _{BRU-NIN}	Clade B, distantly related to HIV-1 _{BRU} (17)	None

perature. After incubation with streptavidin-HRP (Dakopatts) for 1 h followed by wash three times in PBS-Tween, binding of the monoclonal antibodies was visualized as described previously. Titers were estimated as the serum dilution giving 50% reduction of monoclonal antibody binding to coated antigen.

Statistical Analysis

All ELISA titers are expressed as geometric means of arithmetic values and were compared with respect to the incidence and level of serum antibody by Mann-Whitney *U*-test, using StatView SE+ (Abacus Concepts Inc., CA, U.S.A.) computer software. Confidence limits of $\approx 95\%$ for ELISA titers were calculated using Minitab 10 computer software (Minitab Inc., PA, U.S.A.).

RESULTS

To explore the possibility that large oligosaccharides of the CD4-binding domain interfere with the induction of serum antibody response, we immunized animals with wild type gp160 or mutant gp160 (gp160_{A123}), lacking N-glycans at N406, N448, and N463, formulated in iscoms (21,22). This adjuvant system was chosen because immunization with HIV- and SIV-glycoprotein formulations in iscoms generally induces high levels of neutralizing antibodies and protection against infection (29, 30). Two groups of guinea pigs were immunized as shown in Table 1. The animals in group I were primed and boosted with wild type gp160, whereas the animals in group II were primed and boosted with gp160_{A123}. The antibody responses induced by gp160 and gp160_{A123} were analyzed in an ELISA system using gp160 or a synthetic peptide representing the tip of the V3-loop as an antigen (Table 1). Gp160_{A123} induced high antibody titers to HIV-1 gp160 as well as to the principal neutralization loop V3. No significant difference was found between animals immunized with wild type gp160 or gp160_{A123} (Table 1).

The specificity of the antibody responses was analyzed in RIPA using pooled sera from guinea pigs immunized with wild type gp160 or gp160_{A123}. Extracts from r-vaccinia virus-infected, [³H]-GlcN-labeled cells expressing HIV-1 *env* gene products were used as target antigens. Both serum pools pre-

Wt immun.				Mut. immun.				P4D10	
Lys		Sup		Lys		Sup		Sup	
11	17	11	17	11	17	11	17	11	17

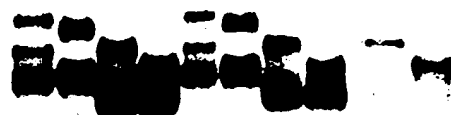


FIG. 1. (RIPA) and subsequent SDS-PAGE of material from [³H]-GlcN-labeled BSC-40 cells, infected with v-J1 or v-J7. Cell lysates (Lys) or culture supernatants (Sup) were included. Pooled sera from animals immunized with wild type (Wt) gp160 or mutant (Mut.) gp160 lacking the three N-linked glycans were analyzed. As a control, supernatants from cells infected with J1 or J7 were precipitated with P4D10, a monoclonal antibody, specific for a linear epitope of the V3 crown. Positions of molecular weight markers of 200K, 100K, and 92K are indicated, as well as positions of wild type gp160, gp120, vaccinia virus hemagglutinin, and an extracellular and soluble non-HIV-related glycoprotein (S).

cipitated gp120 and gp160 equally well, and no difference was found in the amounts of wild type and mutant glycoproteins precipitated by the analyzed sera (Fig. 1). These results indicated there was no preference in precipitation for mutant or wild type envelope glycoproteins irrespective of animals being immunized with gp160_{A123} or with wild type gp160.

To determine if sera from animals immunized with gp160_{A123} were more efficient in neutralizing homologous HIV-1, i.e., lacking the corresponding three N-glycans, we constructed a fully infectious mutant virus clone lacking signals for N-glycosylation at N406, N448, and N463 (Table 2). This virus clone, characterized elsewhere (A. Hemming et al., unpublished observations), had the same infectivity titer as its parent clone. Wild type (HIV-1_{BRU}) and mutant (HIV-1_{BRU-A123}) virus clones were used as targets for determining the neutralizing serum titer of the immunized guinea pigs (Table 3). The immunization efficacy was expressed as the number of immunized animals producing sera with neutralizing titers >80. Sera from animals immunized with wild type gp160 neutralized the homologous wild

TABLE 3. Immunization efficacy of sera from animals immunized with wild type gp160 or gp160_{A123}

Animal group	Immunogen	Efficacy of sera neutralizing target virus ^a			
		HIV-1 _{BRU}	HIV-1 _{BRU-A123}	HIV-1 _{MN}	HIV-1 _{BRU}
I	gp160-iscom	6/9	4/10	1/10	8/10
II	gp160 _{A123} -iscom	4/10	8/10	0/10	2/10

^a The immunization efficacy is expressed as the number of animals producing sera with a neutralizing titer >80.

type HIV-1_{BRU} clone more efficiently than the mutant virus clone, HIV-1_{BRU-A123}. Conversely, sera from animals immunized with gp160_{A123} neutralized the homologous mutant clone HIV-1_{BRU-A123} more efficiently than the wild type virus (Table 3). These results indicated a homologous preference in neutralization capacity of the sera from the immunized animals. A similar neutralization pattern was observed when a closely related virus, HIV-1_{BRU-N308} (see Table 2), was used as target virus (24,31) (Table 3). This virus clone was more susceptible to neutralization by sera from animals immunized with wild type gp160 compared to sera from animals immunized with gp160_{A123} (Table 3).

To analyze whether gp160_{A123} would generate a more broadly neutralizing immune response than the corresponding wild type gp160, a distantly related HIV-1 strain within Clade B, HIV-1_{MN} (17), was used as target virus in neutralization tests designed as noted previously (Table 3). Only 1 of the 10 animals immunized with wild type gp160 developed a significant titer to HIV-1_{MN}, whereas all sera from animals immunized with gp160_{A123} failed to neutralize this strain. This result indicated that elimination of the three N-linked glycans did not broaden the neutralizing response, even among strains within Clade B.

One important question was whether the induction of antibodies to the CD4-binding domain was altered by the gp160_{A123}. The capacity of sera from the animals in groups I and II to block the binding between gp160 and a well-characterized neutralizing human monoclonal antibody against the CD4-binding domain (GP13) (27) was compared (Table 4). A control experiment demonstrated no difference between the affinity of this conformation-dependent monoclonal antibody to wild type gp160 and gp160_{A123} (data not shown). Blocking capacity of sera was expressed as mean antibody titer reduc-

ing the MAbGP13/gp160-binding by 50%. No difference was found between antibodies from animals immunized with wild type gp160 or gp160_{A123} in the capacity to block the MAbGP13-binding to either wild type gp160 or gp160_{A123} (Table 4). Thus, our results indicated that the N-linked glycans at N406, N448, and N463 do not prevent the induction of antibodies to the CD4-binding region.

Because the CD4-binding domain and the V3-loop are situated close together in the three-dimensional conformation of gp120 (32), N-glycans in the CD4-binding region may alter the induction of the neutralizing antibody response to the V3-loop. No difference was found in the antibody response to a synthetic peptide representing the V3-loop, between the two groups of immunized animals (Table 1). However, peptide reactivity of antibodies does not necessarily reflect the levels of virus-neutralizing antibodies (33). We therefore further analyzed the capacity of the sera from the immunized animals to block the binding between gp160 and a well-characterized neutralizing V3 monoclonal antibody, F58/H3 (28), in a competitive ELISA designed as noted earlier (Table 5). No significant difference in the blocking capacity between sera from animals immunized by gp160_{A123} or from wild type gp160 was found. In conclusion, the data suggest that the N-glycans at N406, N448, and N463 in the CD4-binding domain are not involved in protecting neutralization epitopes in the V3-region, neither from pre-existing virus-neutralizing antibodies to V3 nor from induction of such antibodies despite the predicted close localization (32) of these domains in the three-dimensional conformation of gp120.

DISCUSSION

It is well known that N-linked carbohydrates exert two functions in mature viral glycoproteins: (a)

TABLE 4. Titers of guinea pig sera to gp160 and gp160_{A123} in competitive ELISA with MAb (GP13) to CD4 binding domain

Animal group	Immunogen	Blocking capacity ^a			
		gp160		gp160 _{A123}	
		Mean titer ^b	Confidence interval ^c	Mean titer ^b	Confidence interval ^c
I	gp160-iscoms	290	(203-423)	275	(203-473)
II	gp160 _{A123} -iscoms	142	(74-366)	282	(165-674)

ELISA, enzyme-linked immunosorbent assay.

^a Blocking capacity of serum expressed as the serum dilution giving 50% reduction in binding of the αCD4bd-MAb GP13 (27) to wild

type gp160 or gp160_{A123}.

^b All titers are expressed as geometric means.

^c 95% confidence intervals (n = 10) for the ELISA titers are shown within parentheses.

TABLE 5. Titers of guinea pig sera to gp160 and gp160_{A123} in competitive ELISA with MAb (F58/H3) to V3 domain

Animal group	Immunogen	Blocking capacity ^a			
		gp160		gp160 _{A123}	
		Mean titer ^b	Confidence interval ^c	Mean titer ^b	Confidence interval ^c
I	gp160-iscoms	3,700	(2,400–8,500)	3,100	(2,200–6,200)
II	gp160 _{A123} -iscoms	1,300	(860–5,100)	1,500	(910–6,500)

ELISA, enzyme-linked immunosorbent assay.

^a Blocking capacity of serum expressed as the serum dilution giving 50% reduction in binding of the α V3-MAb F58/H3 to wild type gp160 or gp160_{A123}.

^b All titers are expressed as geometric means.

^c 95% confidence intervals (n = 10) for the ELISA titers are shown within parentheses.

continuous stabilization of a functional or at least antigenically appropriate three-dimensional conformation or (b) shielding of otherwise accessible target epitopes from antibodies and other immune effectors. Considering this latter function, protection may not only involve shielding of epitopes from existing antibodies, but also hindering of relevant epitopes from induction of protective immunity. This latter aspect was explored for the CD4-binding domain of gp120 by comparing the immune response of animals immunized with gp160 lacking the N-linked glycans at N406, N448, and N463 to that of animals immunized with the corresponding wild type protein. According to these two described functions of N-linked glycans, one or a combination of two possible scenarios could be expected: (a) demasking, caused by deletion of the three N-linked glycans inducing a significantly broadened antibody response, resulting in an increased range of different neutralizable HIV strains; (b) a carbohydrate-induced conformational change, caused by depletion of the N-linked glycans, resulting in an altered neutralization specificity focused to the mutant virus rather than broadening the neutralization response.

Our data clearly support the latter scenario demonstrating that the carbohydrate manipulation of the immunogen neither broadened the narrow virus-neutralizing capacity nor increased the neutralizing titers of the induced immune response. In fact, the modified immunogen did not even succeed in inducing detectable neutralizing antibodies to HIV-1_{MN} also belonging to Clade B. In contrast, we found a serum antibody response even more focused to a preferred homologous neutralization. These results indicated that wild type and mutant virus exposed at least partly different antigenic determinants to the immune system, affecting the specificity of the induced neutralizing antibodies as well as the sus-

ceptibility to neutralization of the mutagenized virus.

An intriguing question pertains to the molecular mechanism behind this carbohydrate-induced difference in immune response. It is established that the antigenic conformation of gp120 and some other viral glycoproteins is variable due to regulation by presence or absence of peripheral galactose units of complex type N-linked glycans (12,34). Although a linear epitope in the C4 region was found not to be regulated by such galactose residues (35), neutralization epitopes of both the V2 and V3 regions were identified as galactose-dependent epitopes (12,35). Interestingly, two of the three eliminated N-glycans of the present study constitute complex type N-linked glycans, exposing relevant galactose units, as identified in recombinant gp120 expressed in mammalian cell lines (1). It is therefore plausible that elimination of the three N-glycans at N406, N448, and N463 induces a conformational change via galactose-dependent modulation of the three-dimensional structure sufficient to change the immunogenic properties of the glycoprotein without interfering with glycoprotein function in the HIV replication cycle.

Besides the change of the neutralization specificity, virtually no difference between sera from animals immunized with gp160 and gp160_{A123} was found. This includes specificity, as determined in ELISA, to a V3 peptide and homologous and heterologous gp160, but also the capacity of sera to compete with antibodies to the V3 loop or to the CD4-binding region. These latter data are in accordance with previous results that HIV-1_{BRU-A123} is not more susceptible to neutralization by anti-V3 CD4-blocking antibodies than is the corresponding wild type virus (A. Hemming et al., unpublished observations). The fact that differences in the neutralization pattern were noted for the two categories

of sera without corresponding difference in their capacity to compete in vitro with V3- or CD4-blocking antibodies emphasizes that results obtained with purified, monomeric gp160 do not necessarily reflect the details of the biologically relevant situation, where an infectious HIV particle is attacked by neutralizing antibodies to its surface glycoprotein.

One major goal is to construct an improved immunogen exposing highly conserved and immunogenic neutralization epitopes to the immune system, hopefully resulting in high titers of broadly reactive neutralizing antibodies: Would mutants of gp160, lacking either single or combinations of N-linked glycans in other regions than the one analyzed in the present study, fulfill these requirements? That question is, of course, impossible to answer without a more comprehensive analysis of such mutants. Our results indicate a possible danger associated with this strategy: Elimination of N-linked glycans may result in epitope modification rather than in epitope demasking. However, recent results (8), independently confirmed in our laboratory (31), show that an N-linked glycan of the V3 loop is directly involved in epitope protection, and that genetic elimination of this oligosaccharide results in a fully infectious HIV clone, but with strongly increased susceptibility to neutralization by anti-V3-antibodies. We therefore recommend a continued mutational analysis, where different combinations of N-linked glycans are eliminated from gp120 and the consequences of these manipulations in terms of possible quality improvements of immunogen properties are evaluated.

Several recent reports emphasize the difference in epitope presentation between monomeric and oligomeric *env* gene products, where the reactivity of a given antibody with oligomeric but not with monomeric gp120 reflects the capacity of the antibody to neutralize the HIV strain in question (36-39). Consequently, the relative resistance of primary isolates to be neutralized by antibodies to linear as well as conformational epitopes of gp120 is accompanied by low antibody affinity for corresponding oligomeric *env* gene products (39), possibly restricting the use of subunit immunogens based on HIV-1 *env* gene products. However, for antibodies to linear epitopes of the V3 region, we recently showed that the discrepancy between neutralization capacity and oligomer reactivity on one hand and the reactivity toward monomeric gp120 on the other hand are dependent on the existence of one defined

N-linked glycan in the V3 region (40). This means it will be possible at least to expand the display of neutralization epitopes on oligomeric HIV-1 *env* gene products by site-directed elimination of defined glycans although as yet it is too early to conclude whether such manipulations will improve the quality of an immunogen.

Finally, N-linked glycans may also influence the antigen recognition by CD4⁺ T-lymphocytes (41) and priming of HIV-1 gp120-specific CD8⁺ CTL activity (42). Of special relevance for the present paper is the recent finding that presence of defined N-linked oligosaccharide side chains of gp120 in certain cases are crucial also for in vivo priming of T cells, recognizing epitopes in close proximity of such N-linked glycans (43).

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Importance of Hypervariable Regions of HIV-1 gp120 in the Generation of Virus Neutralizing Antibodies

NANCY L. HAIGWOOD, JEFFREY R. SHUSTER, GREGORY K. MOORE,
HELEN LEE, PAUL V. SKILES, KEITH W. HIGGINS, PHILIP J. BARR,
CARLOS GEORGE-NASCIMENTO, and KATHELYN S. STEIMER

ABSTRACT

Variants of the envelope gene of the HIV-SF2 isolate of HIV-1 with deletions of one or more of the hypervariable domains of gp120 were produced in genetically engineered yeast as nonglycosylated denatured polypeptide analogs of gp120. Purified antigens were used to immunize experimental animals to determine whether the removal of hypervariable regions from this type of gp120 immunogen had any effect on (1) the ability of the antigen to elicit virus neutralizing antibodies; and (2) the isolate specificity of the neutralizing antibodies that were elicited. The results of these studies demonstrate that, in addition to the previously identified V3 domain, at least two other hypervariable regions in gp120 are capable of eliciting neutralizing antibodies in experimental animals. However, when all five of the hypervariable regions were deleted, the resulting antigen was no longer capable of eliciting neutralizing antibodies. Finally, the neutralizing antibodies elicited by all of these nonglycosylated antigens were effective against HIV-SF2, the isolate from which the antigens were derived, but were not able to neutralize two divergent isolates, HIV-BRU or HIV-Zr6.

INTRODUCTION

THE ENVELOPE GLYCOPROTEIN gp120 OF HUMAN IMMUNODEFICIENCY virus (HIV-1) is a logical candidate as a subunit vaccine immunogen for this virus. This glycoprotein binds to CD4,¹⁻³ the putative viral receptor, and has been shown to elicit virus neutralizing antibodies in a variety of experimental animals.⁴⁻⁸ Also, gp120 has been shown to bind at least a portion of the HIV-1 neutralizing antibodies present in sera from HIV-1-infected humans.^{8,9} However, sequence analysis of independent HIV-1 isolates has shown that the region of the envelope gene encoding gp120 is characterized by considerable sequence variation.¹⁰⁻¹² This

Chiron Research Laboratories, Chiron Corporation, Emeryville, CA 94608.

variation is clustered in five major regions referred to as hypervariable regions.¹³⁻¹⁶ The possibility that this sequence heterogeneity might complicate efforts to make an effective vaccine employing a gp120 immunogen was recognized immediately.

Human HIV-positive sera from infected asymptomatic individuals are characterized by having antibodies effective in neutralizing a fairly broad spectrum of virus isolates in vitro, suggesting neutralization via conserved epitopes.¹⁷ Studies employing synthetic peptides have demonstrated that conserved^{18,19} as well as variable²⁰⁻²² regions of gp120 can elicit neutralizing antibodies in experimental animals. The identification of such epitopes in conserved regions supports the notion that gp120 might be able to elicit neutralizing antibodies effective against all HIV-1 isolates. However, the marked sequence heterogeneity which occurs in gp120 dramatically influences the specificity of the neutralizing antibodies elicited by complex versions of this antigen. Antibodies raised by all complex gp120 immunogens, including fully glycosylated native versions of gp120 purified from virus-infected cells^{23,24} or produced in genetically engineered mammalian cells,^{4,25} as well as nonglycosylated denatured gp120 antigens produced in genetically engineered microorganisms,²² have been shown to be capable of neutralizing primarily the virus strain from which the gp120 antigen was derived. Thus, despite the fact that conserved epitopes capable of eliciting neutralizing antibodies are present in these immunogens, they are apparently not seen in the context of larger gp120 antigens. The fact that antibodies specific for these conserved epitopes are not detected in sera from HIV-infected humans¹⁸ further supports this conclusion. Finally, human gp120-specific antibodies purified by affinity chromatography using both fully glycosylated gp120 as well as nonglycosylated versions of the molecule have also been shown to exhibit a specificity for the strain from which the antigen was derived,^{8,9} although this specificity is not as restricted as the neutralizing response elicited by purified gp120 antigens in experimental animals.

Several studies have been performed in an effort to more precisely map the epitopes within complex gp120 immunogens that are responsible for generating neutralizing activity. No convincing evidence for neutralizing antibodies to conformational epitopes found within either conserved or variable regions of gp120 has been obtained. To date, only one region of gp120 has been shown to be involved in eliciting neutralizing antibodies. This region is within the third hypervariable domain of gp120, commonly referred to as V3, and consists of a stretch of approximately 32 amino acids bounded by cysteine residues that most likely form a disulfide bond in the native molecule.²⁰⁻²² The epitope within V3 that elicits neutralizing antibodies appears to be a linear sequence of amino acids that is accessible in native glycosylated versions of gp120 as well as denatured nonglycosylated gp120 immunogens.²² Furthermore, synthetic peptides corresponding to this epitope can also elicit neutralizing antibodies.²¹ To date, there has been no other region of gp120 shown to elicit neutralizing antibodies when complex immunogens displaying multiple gp120 determinants have been used.

The studies described in this report were undertaken in an attempt to design a gp120 immunogen that would be capable of eliciting an immune response to conserved neutralizing epitopes. Based on a hypothesis by Coffin²⁶ that the hypervariable regions of gp120 might be highly immunogenic, and therefore, may serve as decoys distracting the immune system from the more conserved regions, we reasoned that their removal might unmask other epitopes that normally are not seen when more complex versions of gp120 are used as immunogens. In order to test this hypothesis, we have produced a series of gp120 hypervariable region deletion variants.

We describe the results of immunogenicity studies with nonglycosylated denatured versions of these deletion variants produced in genetically engineered yeast. We show that the removal of the hypervariable regions of gp120, either singly or in concert, does not result in an immune response to conserved neutralizing epitopes in this type of immunogen. As observed with antibodies to the full-length version of gp120, antisera to deletion mutants that exhibited neutralization did not neutralize the other HIV-1 isolates tested. However, we did observe that hypervariable regions of gp120 other than V3 were also capable of eliciting neutralizing antibodies when presented in the context of a complex immunogen. Furthermore, antibodies directed to other hypervariable regions, like those to the V3 region, also exhibited specificity for the isolate from which the antigen was derived. Finally, since nonglycosylated gp120 with all five of the hypervariable regions deleted was not able to elicit any neutralizing activity, we conclude that at least one hypervariable region in this type of immunogen is required to elicit neutralizing antibodies.

MATERIALS AND METHODS

Viruses and virus culture methods

The three isolates of HIV-1 used in this study were HIV-SF2 (originally referred to as ARV-2²⁷; provided by J. A. Levy, University of California, San Francisco, California; HIV-BRU (originally LAV-1²⁸); from F. Barre-Sinoussi, Pasteur Institute, Paris, France; and HIV-Zr6¹⁹ obtained from A. Srinivasan, Centers for Disease Control, Atlanta, Georgia. Viruses were propagated in HUT-78 cell²⁹ obtained from the American Type Culture Collection, Rockville, Maryland.

Stocks of each isolate were prepared from acutely infected cultures of HUT-78 cells, 7–10 days after inoculation with virus at a time when 60–80% of the cells in the culture showed evidence of infection (i.e., cytopathology and/or syncytium formation). Clarified culture supernatants from virus-infected cells were frozen at -80°C in aliquots and were thawed just prior to use in the neutralization assay (see below).

Mutagenesis and expression of HIV gp120 in yeast

The envelope gene encoding gp160 of HIV-SF2 was engineered for expression of gp120 sequences by the introduction of a stop codon following Arg509 at the gp120-gp41 processing site. The 5' end of the gene was modified to insert an NheI restriction endonuclease site 5' to the sequences encoding Glu31, so that the natural signal sequence could be removed for intracellular expression in yeast. For deletion, in vitro site-directed mutagenesis was performed on M13 template containing the entire gp120 coding sequence. Oligomers were 36 bp, with 18 bp on each side of the desired deletion. DNA sequencing was used to verify the changes. The expression vector pHL15 was used in the expression of hypervariable deletion proteins. Expression was directed with a transcriptionally regulatable hybrid ADH2/GAPDH promoter³⁰ and a host strain genetically engineered for the overproduction of its transcriptional activator protein.³¹ Genes were engineered for intracellular expression in order to obtain nonglycosylated recombinant polypeptides. Coding regions for the different *env* proteins were substituted as NheI-SalI DNA fragments into the pHL15 vector. The DNAs for the deletion variant proteins expressed in yeast encode Met-Ala-Ser-Glu 31—at their N terminus. Wild-type SF2 gp120 in yeast, referred to as *env* 2-3, was engineered at the 5' and 3' ends using synthetic oligomers to encode Ile26 through Ala510.^{32,33} Proteins representing the two halves of the gp120 coding sequence were made by utilizing a BglII site at position 826 that essentially bifurcates the gene at amino acid Arg276 to make *env*-1 (aa 26-276),³² and *env*-4 (aa 272 through 509). The N-terminal amino acid sequence of *env*-4 is Met Glu272 Val273 Val274—and the C terminus is Arg 509. The expression plasmid constructs were transformed into *Saccharomyces cerevisiae* strains and highly expressing colonies were isolated.

Purification of recombinant gp120 antigens

All of the HIV-1 envelope antigens were purified as described previously.³² The resulting antigens were all greater than 90% pure as judged by Coomassie staining of sodium dodecyl sulfate-polyacrylamide gels (see results).

Immunization of animals with purified recombinant antigens

Female Hartley guinea pigs were given primary immunizations of 50 μg of purified antigen in MTP-PE⁸ in the footpad and then received monthly booster doses of 50 μg of antigen in adjuvant by the same route. The animals were bled prior to the primary immunization (prebleed) and one week following each booster dose of antigen. Goats were injected intramuscularly with 1 mg primary doses of antigen in complete Freund's adjuvant (CFA) and given monthly intramuscular booster doses of 0.5 mg of antigen in incomplete Freund's adjuvant (IFA). Goats were bled prior to the primary immunization (prebleed) and two weeks following each booster immunization.

Assays

Virus neutralization assay. The assay procedure was similar to the protocol described previously.^{7,8,34} Sera were heat inactivated for 60 min at 56°C and then diluted serially in culture medium (RPMI-1640 with 10% heat-inactivated fetal bovine serum). Equal volumes of diluted serum and virus inoculum were mixed and incubated for 1 h at room temperature. Following incubation, 0.1 ml of the mixture was added to 1 ml cultures of HUT-78 cells (1×10^4 cells/ml) in 24-well microtiter plates. After 7 days, the cells were pelleted, the supernatants were discarded, and the pelleted cells were lysed in phosphate-buffered saline (PBS) containing 1% Triton X-100. Infection was monitored by measuring the intracellular levels of the major HIV-1 core protein, p25^{gag}, in a capture enzyme-linked immunosorbent assay (ELISA; protocol described below). The inoculum for each virus isolate was adjusted to yield approximately 5–10% infection of the HUT-78 cells in the culture and 50–100 ng/ml of intracellular p25^{gag} during the 7-day assay period. This inoculum corresponds to approximately 10^3 infectious virus particles. Sera were judged to be positive in the assay if they inhibited p25^{gag} antigen production by 60% (or greater) at the most concentrated dilutions of 1:10 and 1:20 for goats and guinea pigs, respectively. The neutralization titers reported are the reciprocal of the serum dilution in the virus and serum mixture that reduced the production of p25^{gag} by 50%. Each serum dilution was tested in duplicate and the results averaged.

Enzyme-linked immunosorbent assays (ELISA): Titration of serum antibodies. Levels of antibodies specific for the various recombinant antigens in sera from immunized animals were determined by a modification of the indirect ELISA described previously.^{35,36} Purified antigens were adsorbed to microtiter plates at 2 µg/ml. Conjugates used were horseradish peroxidase-conjugated rabbit antibodies specific for guinea pig immunoglobulin (Boehringer Mannheim Biochemicals, cat. no. 605360, diluted 1/2000) or swine antibodies specific for goat immunoglobulin (Boehringer Mannheim Biochemicals, cat. no. 605275, diluted 1/2000) for assaying guinea pig and goat sera, respectively. The substrate was 2,2-azino-bis(3-ethylbenzthiazoline sulfonic acid) [ABTS].

Capture ELISA for HIV-1 p25^{gag} antigen. The protocol for quantifying p25^{gag} antigen levels in lysates from the neutralization assay was as follows: dilutions of lysates were added to microtiter plates that had been coated with 5 µg/ml of the immunoglobulin fraction from ascites of 76C, a mouse monoclonal antibody specific for HIV-1 p25^{gag}.³⁶ Following incubation at 37°C for 2 h, the plates were washed and then a rabbit polyclonal antiserum raised against purified disrupted HIV-SF2 virus (diluted 1/3000) with a high titer of antibodies to p25^{gag} was added to the wells for 1 h followed by a 45-min incubation with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin (Tago, diluted 1/2000). As in the titration procedure above, the substrate was ABTS. The levels of p25^{gag} were calculated based on a standard curve generated using purified recombinant p25^{gag} produced in *Escherichia coli*.³⁶

RESULTS

Design of gp120 hypervariable deletion mutants

The sequence variation of the HIV-1 envelope gene is clustered in five major hypervariable regions in gp120, with the arrangement of constant (C) and variable (V) domains following the pattern C1-V1-V2-C2-V3-C3-V4-C4-V5-C5 (Fig. 1). In the HIV-SF2 isolate, the variable regions correspond to amino acids 131–154 (V1), 156–198 (V2), 292–365 (V3), 388–414 (V4), and 456–465 (V5). The hypervariable sequences were assigned on the basis of previously published amino acid sequence comparisons of HIV-1 isolates that delineated the approximate boundaries of variable and constant regions.^{13,37} Deletion mutants were designed to retain Cys residues, based on the idea that the hypervariable regions may exist as loops between conserved Cys residues. Two deletions were made within the first and second hypervariable regions (amino acids [aa] 131–154 and 156–198); one deletion within the third hypervariable region (aa 300–332); one deletion within the fourth (aa 388–414); and one deletion within the fifth hypervariable regions (aa 456–463). These deletions were engineered into expression vectors for the expression of polypeptides representing the full length or subregions of gp120. Figure 1 shows schematically the recombinant wild-type and deletion variants used in this study. Variants were named for their corresponding deletions, *env* 2-3 D3 for

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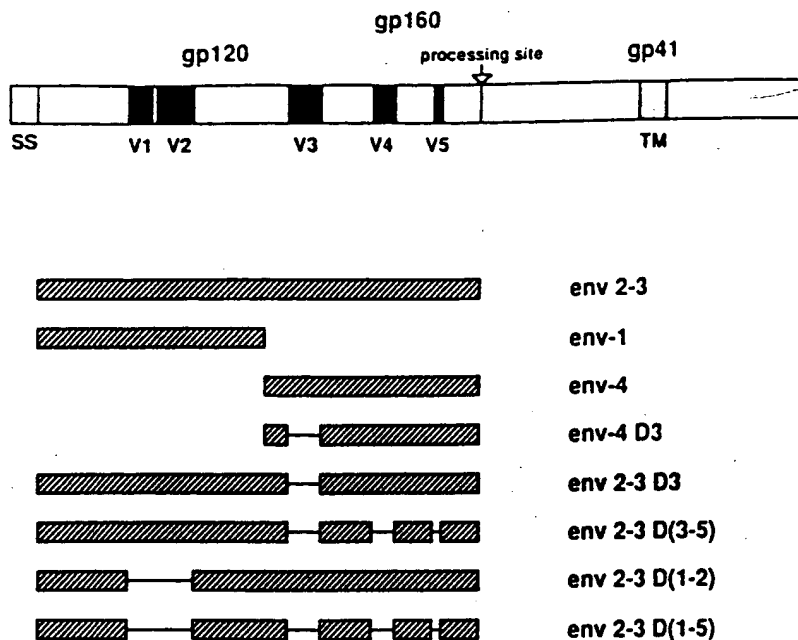


FIG. 1. Schematic diagram of the HIV-1 envelope gene showing locations of hypervariable regions and variants produced in yeast. The *env* gene encodes gp160, which is processed into gp120 and gp41 at the processing site shown. Constant domains are shown in white and variable sequences in black. SS = signal sequence; TM = transmembrane domain. The bars shown below the schematic diagram delineate the regions that were expressed in variants of gp120 in yeast, and deleted regions are shown with thin connecting lines. Deletion variants were named to correspond with the variable regions deleted. Specific amino acids encoded by *env* 2-3, *env*-1, and *env*-4 are described in Materials and Methods.

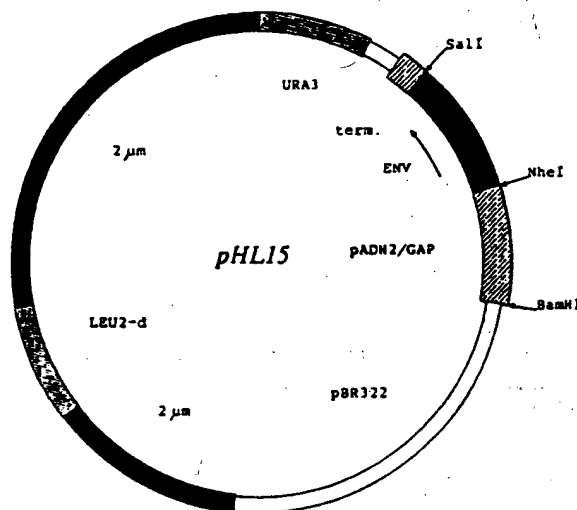


FIG. 2. Schematic diagram of expression plasmid pHL15 for the production of recombinant HIV-1 gp120 antigens in yeast. pHL15, 15.6 kb. The SF2 gp120 coding sequences (aa Glu31 through Arg509) are labelled ENV with an arrow denoting the direction of transcription. The regulable hybrid ADH2/GAPDH promoter, pADH2/GAP, and terminator (term), are shown hatched. URA3 and LEU2-d denote genes encoding uracil and leucine selectable markers, 2 μ in black denotes sequences from the yeast 2 micron plasmid. pBR322 sequences in white allow replication and ampicillin resistance selection in *E. coli*. Regions are drawn to scale.

the full-length gp120 polypeptide minus the V3 region, etc. The expression vector utilized for full-length gp120 expression in yeast is shown schematically in Figure 2. All polypeptides were expressed intracellularly in yeast and were therefore not glycosylated; antigens were denatured during purification.

Characterization of recombinant gp120 antigens

Expression of the wild-type and hypervariable region deletion variants of HIV-SF2 gp120 was confirmed by immunoblot analysis with human HIV-1 antibody-positive sera. Each antigen was purified to >90% purity as described in the Methods section. The mobility and purity of each antigen is illustrated in Figure 3, panel A. Below, in panel B, is shown the immunoreactivity of each of these antigens with human HIV antibody-positive sera. The apparent molecular weight of the nonglycosylated wild-type HIV-SF2 gp120, referred to as SF2 *env* 2-3, was approximately 55 kD, consistent with the size predicted from the amino acid sequence. Furthermore, the hypervariable deletion variants migrated on gels as predicted from the sizes of their deletions. In addition to the full-length gp120 analogs, polypeptides corresponding to the amino- (*env*-1,

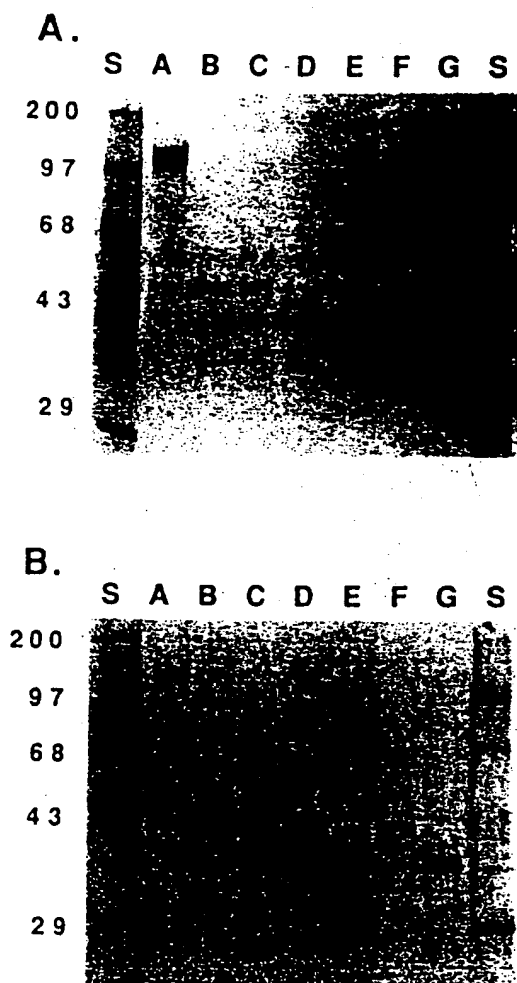


FIG. 3. Coomassie-stained sodium dodecyl sulfate (SDS) gel and Western blot showing purified gp120, *env* 2-3, and *env* 2-3 variants. 8% Laemmli denaturing gel showing 200 ng CHO cell-derived gp120, A; 100 ng *env* 2-3 SF2, B; 100 ng *env* 2-3 HTLVIII, C; 100 ng *env* 2-3 D3, D; 100 ng *env* 2-3 D(1-2), E; 100 ng *env* 2-3 D(3-5), F; 100 ng *env* 2-3 D(1-5), G. S refers to high molecular weight standards, and the sizes are noted in kD. Top panel is a Coomassie-stained gel; bottom panel is a blot of these samples developed with human HIV-positive immunoglobulin.

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aa 26-276) and carboxyl (*env*-4, aa 272-509) terminal halves of gp120, as well as the *env*-4 molecule with the V3 region deleted, were also produced. These nonglycosylated antigens migrated as predicted on SDS polyacrylamide gels, reacted as expected with pooled human HIV antibody-positive sera, and were purified to homogeneity (not shown). All full-length wild-type and deletion variant antigens showed equivalent reactivity to the human sera tested. Of note is the reactivity of deletion variant D(1-5) with all human HIV⁺ antibody-positive sera tested, despite the lack of variable sequences in the antigen. All proteins were tested for binding to recombinant, soluble CD4, and binding was at least 200-fold reduced relative to equimolar amounts of recombinant native, glycosylated gp120 (data not shown).

Effects of deletion of specific hypervariable regions on the generation of neutralizing antibodies in experimental animals

The various wild-type and hypervariable deletion mutants of gp120 produced in yeast were purified following denaturation and extraction and were used to immunize guinea pigs and goats. Antisera were titrated by indirect ELISA to measure the antigen-specific antibody response. The sera were tested for *in vitro* neutralization of the HIV-SF2 and HIV-BRU virus isolates, and in certain cases, also the HIV-Zr6 isolate. Results with sera from animals immunized with wild-type antigens are shown in Table 1, and results with variant antigens are summarized in Table 2. Examples of neutralization assay results with selected groups of animals are shown graphically in Figure 4. Note that the data for then *env* 2-3 immunized guinea pigs shown in Tables 1 and 2 are from separate experiments. We occasionally observe groups or individuals of *env* 2-3-immunized animals that show low titers of neutralizing activity as observed for the guinea pigs in Table 2. These data were included because they were the control animals immunized in parallel with the other guinea pig immunizations shown in the table. We have no explanation for the variation from experiment to experiment. However, it should be remembered that these are outbred animals and therefore would be unlikely to show uniform responses.

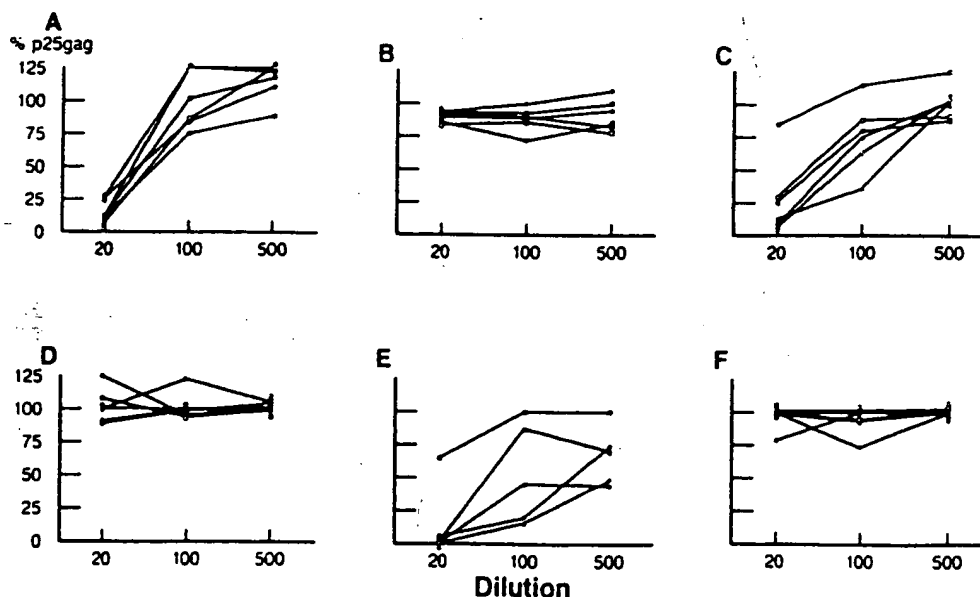


FIG. 4. Neutralization of HIV-1 by antisera from guinea pigs hyperimmunized with wild-type and hypervariable deletion mutants of gp120 produced as nonglycosylated antigens in yeast. Shown are the results of neutralization assays with antisera from guinea pigs (see Table 2) immunized with *env* 2-3 (Panels A and B), *env* 2-3 D(1 + 2) (Panels C and D), *env* 2-3D3 (Panel E), and *env* 2-3 D(1-5) (Panel F). The results shown in A, C, E, and F are from HIV-SF2 and B and D are from HIV-BRU neutralization assays.

TABLE 1. NEUTRALIZATION OF HIV-1 ISOLATES BY ANIMAL ANTISERA TO WILD-TYPE RECOMBINANT gp120 ANTIGENS PRODUCED AS NONGLYCOSYLATED DENATURED POLYPEPTIDES IN YEAST^a

Antigen	Isolate of origin ^b	Animal immunized ^c	Animal number	ELISA titer ^d			Neutralization titer		
				SF2 ^e env 2-3	IIIB ^e env 2-3	Zr6 ^e env 2-3	SF2	BRU ^e	Zr6
env 2-3	SF2	Guinea pig	839	365,400	595,000	140,100	^f >500	^h < 20	< 20
			840	332,200	324,800	57,000	> 500	< 20	< 20
			841	305,400	143,000	94,200	> 500	< 20	< 20
			842	471,600	256,100	99,900	> 500	< 20	< 20
env 2-3	SF2	Goat	101	166,100	171,100	51,300	700	< 10	ⁱ nt
			102	182,200	140,400	44,100	1,250	< 10	nt
env 1	SF2	Guinea pig	1873	53,300	17,100	6,500	60	< 20	nt
			1874	12,200	3,800	1,700	90	< 20	nt
			1875	97,200	21,200	13,700	> 500	< 20	nt
			1876	100,900	17,800	10,200	> 500	< 20	nt
			1877	321,500	56,900	24,600	> 500	< 20	nt
			1878	17,300	4,000	2,100	500	< 20	nt
env 1	SF2	Goat	490	29,500	26,500	6,800	700	< 10	< 10
			491	14,200	37,200	6,200	> 1,250	< 10	< 10
env 4	SF2	Guinea pig	1879	17,300	28,900	19,200	90	< 20	nt
			1880	64,200	86,200	38,700	> 500	< 20	nt
			1881	44,100	71,100	36,600	> 500	< 20	nt
			1882	81,400	112,800	47,200	> 500	< 20	nt
			1883	42,400	59,300	31,900	> 500	< 20	nt
			1884	19,800	38,800	25,000	> 500	< 20	nt
env 4	SF2	Goat	491	80,000	38,400	nt	> 1,250	< 10	< 10
			492	90,000	108,700	nt	250	< 10	< 10
			493	68,000	nt	nt	130	< 10	< 10
env 2-3	IIIB	Guinea pig	2523	22,000	52,200	17,800	< 20	130	nt
			2524	45,600	146,200	41,200	< 20	500	nt
			2526	24,800	101,100	24,100	< 20	175	nt
			2527	37,800	110,200	37,000	< 20	175	nt
			2528	18,000	55,500	20,500	< 20	125	nt
Positive control ⁱ	?	Human	20058	50,000	12,184	nt	4,820	> 250	50
	?	Human	20028	54,231	37,645	nt	4,500	130	nt
Negative control ⁱ	none	Human	NHS	< 100	< 100	< 100	< 20	< 20	< 20

^a Antisera to each antigen was raised in goats and/or guinea pigs as described in the Methods section. Note that none of the prebleed sera showed any neutralization of HIV-SF2 or HIV-BRU nor did they give a signal on either env 2-3 ELISA.

^b HIV-1 strain from which gp120 sequences were derived.

^c Data shown are from guinea pig sera collected after the fourth or fifth immunization; goat sera collected following the tenth or eleventh immunization.

^d Antibody titers were measured by ELISA using plates sensitized with env 2-3 from HIV-SF2 or HIV-IIIB.

^e Abbreviations for virus strains were SF2, IIIB, and BRU for HIV-SF2, HIV-IIIB and HIV-BRU, respectively.

^f "nt" = not tested.

^g Neutralization titers listed as ">x" when complete (> 90%) neutralization was observed at the most dilute concentration tested.

^h No neutralization was observed at the most concentrated dilution tested.

ⁱ Positive controls were human HIV-1 antibody-positive serum specimens and the negative control was pooled human sera (HIV seronegative) obtained from Medical Specialties Laboratories, Boston MA.

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TABLE 2. NEUTRALIZATION OF HIV-1 ISOLATES BY ANIMAL ANTISERA TO HYPERVARIABLE DELETION VARIANTS OF HIV-SF2 gp120^a

Antigen	Isolate of origin ^b	Animal immunized ^c	Animal number	ELISA titer ^d		Neutralization titer	
				SF2 ^e env 2-3	IIIB ^e env 2-3	SF2	BRU ^e
env 2-3	SF2	Guinea pig	2916	377,500	413,500	60	< 20
			2917	173,400	109,000	50	< 20
			2918	204,800	55,500	30	< 20
			2919	301,900	97,800	30	< 20
			2920	703,500	315,100	40	< 20
			2921	14,800	118,200	40	< 20
env 2-3 D3	SF2	Guinea pig	2477	588,200	136,900	50	< 20
			2478	86,900	140,200	> 500	< 20
			2479	396,200	344,700	275	< 20
			2480	5,100	11,300	< 20	< 20
			2481	119,400	97,300	> 500	< 20
env 2-3 D3	SF2	Goat	2619	29,600	50,900	20	< 10
			2620	23,100	34,000	300	< 10
env 4 D3	SF2	Guinea pig	2471	21,800	57,500	125	< 20
			2472	27,300	63,000	> 500	< 20
			2473	31,800	132,700	< 20	< 20
			2474	64,200	195,100	< 20	< 20
			2475	10,800	53,800	< 20	< 20
			2476	6,300	17,300	> 500	< 20
env 2-3 D(3-5)	SF2	Guinea pig	2489	395,200	61,700	> 500	< 20
			2490	79,500	50,600	25	< 20
			2491	100,500	29,800	45	< 20
			2492	62,800	40,100	25	< 20
			2493	93,000	44,600	< 20	< 20
			2494	194,300	222,400	30	< 20
env 2-3 D(1-2)	SF2	Guinea pig	2910	175,700	135,700	< 20	< 20
			2911	128,300	91,100	55	< 20
			2912	147,100	180,800	70	< 20
			2913	124,100	149,300	150	< 20
			1914	478,900	606,200	40	< 20
			2915	174,900	285,600	35	< 20
env 2-3 D(1-5)	SF2	Guinea pig	2483	183,100	78,300	< 20	< 20
			2484	74,200	101,400	< 20	< 20
			2485	80,200	58,800	< 20	< 20
			2486	59,300	34,100	< 20	< 20
			2487	119,400	106,200	< 20	< 20
			2488	213,700	105,800	< 20	< 20
env 2-3 D(1-5)	SF2	Goat	2621	16,300	33,700	< 10	nt
			2522	62,200	80,500	< 10	nt

^aSee Table 1 for footnotes; refer to data in Table 1 for positive and negative human serum controls.

Antisera to the full-length wild-type nonglycosylated versions of gp120 (env 2-3) from both the HIV-SF2 and HIV-IIIB isolates exhibited neutralization of the virus isolate from which the recombinant antigen was derived (Table 1). However, no cross-neutralization of the other isolates tested was observed; antisera to HIV-SF2 env 2-3 did not neutralize HIV-BRU or HIV-Zr6 virus, and antisera to HIV-IIIB env 2-3 did not neutralize HIV-SF2 virions. These results are consistent with observations that have been reported by others.^{9,24} Also shown in Table 1 are data obtained with antisera to env-1 and env-4, polypeptides

corresponding to the amino and carboxyl-terminal halves, respectively, of HIV-1 gp120. Antisera to both of these molecules were able to neutralize HIV-SF2 in vitro. This activity was isolate-specific, since no neutralization of HIV-BRU was seen with antisera to either molecule. Thus isolate-specific neutralization can be elicited by epitopes in the amino-terminal half of gp120, as well as the carboxyl terminal domain.

Assays of the neutralizing activity of sera from animals immunized with hypervariable region deletion variants (Table 2) illustrate the complexity of the isolate-specific neutralizing antibody response to nonglycosylated denatured gp120 antigens. Neutralizing antibodies specific for HIV-SF2 were elicited by *env* 2-3 D3, *env* 2-3 D(1-2), and *env* 2-3 D(3-5). Since *env* 2-3 D3 and *env* 2-3 D(3-5) both have an intact amino-terminal region, they might be expected to elicit antibody responses similar to *env*-1. Following the same line of reasoning, *env* 2-3 D(1-2) should give a response similar to *env*-4. The data presented in Table 2 show that the responses to the three antigens were as predicted. However, when the *env*-4 variant missing V3 (*env*-4 D3) was tested, it also elicited HIV-SF2 neutralizing antibodies. Therefore, the V3 region was not the only domain in the carboxyl terminal half of gp120 that could elicit neutralizing antibodies. Finally, when antisera to *env* 2-3 D(1-5), the variant from which all five hypervariable regions were deleted, was tested, no HIV-SF2 neutralizing antibodies were detected, even though this protein was highly immunogenic (see ELISA data). The fact that not all of the animals in each group of guinea pigs immunized with the deletion variants showed evidence of neutralizing activity should be pointed out. While we have no explanation for this observation, it may reflect differences in responsiveness among outbred populations.

DISCUSSION

We have examined the consequences of deletion of defined hypervariable sequences from gp120 on the immunogenicity and capacity of denatured nonglycosylated envelope antigens to generate neutralizing activity in experimental animals. Despite the high sequence diversity among isolates of HIV-1, the location of hydrophilic regions of potential antigenicity is remarkably conserved between strains.³⁸ The deletions constructed in this study were designed to remove variable sequences bearing hydrophilic regions that might serve as immunodominant epitopes, as suggested by Coffin.²⁶ One aim of this study was to test whether antigenic competition³⁹ was limiting the humoral immune response to neutralizing epitopes within the conserved domains of gp120. If this were true, then such epitopes might be made more visible to the immune system by the removal of the more dominant epitopes located in hypervariable regions of the molecule. However, our results indicate that the removal of hypervariable regions of gp120, either singly or in concert, did not result in an antibody response to putative conserved neutralizing epitopes, at least in the context of the type of immunogen utilized here.

We also show here that neutralizing antibodies can be elicited by several hypervariable domains of gp120 not previously identified as targets of such antibodies. While there is variation in neutralization titers of animals within groups, the guinea pig and goat data concur (Tables 1 and 2). We attribute differences in the responses of these outbred animals to their genetic backgrounds. Studies from a number of laboratories have shown the importance of the immunodominant epitope(s) located within the V3 hypervariable region in eliciting isolate-specific neutralizing activity.²⁰⁻²² From this study, it is apparent that epitopes located within hypervariable regions other than V3 are capable of eliciting isolate-restricted neutralizing responses in both goats and guinea pigs, as summarized in Table 3. There is at least one such epitope in the amino-terminal half of gp120 (V1 or V2), since *env*-1 and *env*-2-3 D(3-5) can both elicit neutralizing antibodies. Because the *env*-4 molecule with the V3 region deleted elicits neutralizing activity, there must be at least one other region in the carboxyl terminal half of gp120 that can generate neutralizing antibodies. This epitope could be in either the V4 or V5 regions of gp120. The relative importance of these newly identified neutralization epitopes has not been established. Furthermore, it is not known whether the relatively high sensitivity of HIV-SF2 to neutralization in vitro^{7,17} is allowing detection of neutralization that might be difficult to observe with other isolates less sensitive to neutralization. Finally, our results do not rule out the possibility that antigenic competition involving the V3 domain might limit the response to other neutralizing epitopes encoded by variable sequences.

Because the variant *env*-2-3 D(1-5) lacking all amino acids from hypervariable domains does not elicit neutralizing activity, at least one variable region is necessary to generate measurable neutralizing activity in

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TABLE 3. SUMMARY OF THE RESULTS OF VIRUS NEUTRALIZATION ASSAYS WITH SERA FROM GUINEA PIGS AND GOATS IMMUNIZED WITH VARIOUS RECOMBINANT HIV ANTIGENS

Yeast antigen ^a	Description	V regions deleted	V regions remaining	Virus neutralization	
				SF2	BRU
<i>env</i> 2-3	Full length gp120	None	1,2,3,4,5	+	-
<i>env</i> 2-3 (HTLVIIIIB)	Full length gp120 (HTLVIIIIB)	None	1,2,3,4,5	-	+
<i>env</i> 1 ^b	Amino terminal half of <i>env</i> 2-3	(3,4,5) ^c	1,2	+	-
<i>env</i> 4 ^c	Carboxyl terminal half of <i>env</i> 2-3	(1,2)	3,4,5	+	-
<i>env</i> 4 D3	<i>env</i> 4 deletion	(1,2),3	4,5	+	-
<i>env</i> 2-3 D(1-2)	<i>env</i> 2-3 deletion	1,2	3,4,5	+	-
<i>env</i> 2-3 D3	<i>env</i> 2-3 deletion	3	1,2,4,5	+	-
<i>env</i> 2-3 D(3-5)	<i>env</i> 2-3 deletion	3,4,5	1,2	+	-
<i>env</i> 2-3 D(1-5)	<i>env</i> 2-3 deletion	1,2,3,4,5	none	-	-

^a All antigens *except* the HTLV-IIIIB versions of *env* 2-3 were produced from the HIV-SF2 gp120 coding region of *env*.

^b *Env* 1 corresponds to amino acids 28-276 of HIV-SF2 gp120 produced in yeast.

^c *Env* 4 is amino acids 274-510 of HIV-SF2 gp120 produced in yeast.

^d The deletions are D1 (aa 131-154), D2 (aa 156-198), D3 (aa 300-332), D4 (aa 388-414), and D5 (aa 456-463).

^e Regions in parentheses are outside of the coding region included in the portion of *env* expressed.

this system. These results are in contrast to those of Ho and colleagues,^{18,19} which show that constant epitopes can elicit neutralizing activity when presented as peptides. Neutralizing epitopes have also been mapped to the gp41 region of the envelope glycoprotein using synthetic peptides as immunogens.⁴⁰⁻⁴³ However, full-length gp160 produced in baculovirus does not appear to elicit neutralizing antibodies to these conserved gp41 epitopes.⁴⁴ This underscores the potential differences between peptides and more complex immunogens. By simply removing hypervariable regions of gp120, no constant region epitopes that can function to generate neutralizing activity have been exposed.

All deletion variants described in this study with the exception of *env*-1 retain all of the sequences known to be involved in binding to CD4, yet none bind to CD4. The neutralization of HIV-1 by monoclonal antibodies directed against the CD4 binding site has been reported.⁴⁵ No antibodies effective in virus neutralization were generated with *env* 2-3 D(1-5), which bears all known linear epitopes involved in CD4 recognition. Since the antigens used in this study are denatured and nonglycosylated, their conformation differs from native, glycosylated gp120 in the virion. Thus, their lack of binding to CD4 is not surprising. These results, taken together, strongly suggest the necessity for the envelope glycoprotein to be in a native state in order to generate neutralizing immunity against the CD4 binding site or other conformational epitopes.

The immunization of test animals with denatured, nonglycosylated versions of gp120 in this study elicited clear neutralization against the HIV-SF2 isolate and no detectable neutralization of virus isolates HIV-BRU or HIV-Zr6. These results are consistent with other studies using denatured, nonglycosylated antigens,⁵ as well as studies using native, glycosylated gp120.²⁴ Although there is cross-neutralization of certain diverse HIV-1 isolates by human antibodies affinity purified with HIV-SF2 *env* 2-3,⁸ we show here that immunization with this same antigen does not consistently elicit antibodies that exhibit the same pattern of neutralization of HIV-1 isolates. The low titer cross neutralization of HIV-BRU by *env* 2-3 SF2 antisera that was previously described⁸ has not been reproducibly observed. The inability to unmask neutralizing epitopes in constant regions of gp120 by deletions of hypervariable domains suggests that either these epitopes are nonexistent, or that they cannot be presented by a denatured nonglycosylated form of the antigen. We have initiated similar studies with certain of these hypervariable region deletion variants produced as glycosylated antigens in mammalian cells to compare the responses.

The challenge of vaccine development for HIV-1 centers around overcoming the problem of sequence diversity and the proposed ability of HIV-1 isolates to escape neutralization via sequence changes. This

phenomenon, which has been described for visna virus, another lentivirus,⁴⁶ may make the use of a recombinant gp120 antigen from a single isolate of HIV-1 of limited utility. If the other hypervariable regions of gp120 that we have shown capable of eliciting neutralizing antibodies in animals can be shown to elicit neutralizing antibodies in humans, it might be possible to design a single gp120 hybrid molecule with variable regions from diverse isolates. Such an antigen might be effective in generating neutralizing activity against a broad spectrum of HIV-1 isolates.

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Address reprint requests to:

Nancy L. Haigwood

Senior Scientist

Virology Department

Chiron Corporation

4560 Horton Street

Emeryville, CA 94508

Short Communication

Immunogenicity of DNA Vaccines Expressing Human Immunodeficiency Virus Type 1 Envelope Glycoprotein with and without Deletions in the V1/2 and V3 Regions

SHAN LU,¹ RICHARD WYATT,² JOAN F.L. RICHMOND,³ FARAH MUSTAFA,³ SHIXIA WANG,¹ JIAYU WENG,³ DAVID C. MONTEFIORI,⁴ JOSEPH SODROSKI,² and HARRIET L. ROBINSON³

ABSTRACT

DNA vaccines that express the human immunodeficiency virus type 1 HXB-2 envelope glycoprotein (Env) with or without deletions of the major variable regions V1/V2 and V3 were tested for the ability to raise enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody in New Zealand White (NZW) rabbits. Three forms of the Envs were examined: gp120, the surface (SU) receptor-binding domain; gp140, the entire extracellular domain of Env; and gp160, the complete form of Env. For the forms of Env containing the variable regions, the gp120-expressing DNA plasmid was more immunogenic than the gp140- or gp160-expressing DNA plasmids. Removing the V1/2 and V3 variable regions increased the immunogenicity of the gp140- and gp160-expressing DNAs. Deletion of the variable regions also resulted in antibody responses against determinants that were not presented by the forms of Env containing the variable regions. Despite the improved immunogenicity, removing the V1/V2 and V3 domains did not improve the ability of Env to raise neutralizing antibodies. These results suggest that increasing the exposure of internal structures of Env that include the CD4-binding site does not necessarily result in the generation of better neutralizing antibody.

THE ENVELOPE GLYCOPROTEIN (Env) of HIV-1 is the primary target for neutralizing activity. The *env* gene of HIV-1 encodes a protein of ~850 amino acids. Extensive glycosylation of the Env precursor protein produces gp160, the major form of the *env* gene product detected in infected cells.^{1,2} Cleavage of gp160 yields the N-terminal receptor binding SU subunit, gp120, and the C-terminal transmembrane (TM) subunit gp41.³ In the mature form, the Env glycoprotein is a trimeric complex of gp120-gp41 monomers that are held together by noncovalent bonds.⁴⁻⁷ The gp41 glycoprotein serves as the transmembrane anchor for the oligomeric Env.⁸ The structures required to form the oligomeric complex are within the extracellular domain of Env, and include the leucine zipper sequences of the gp41 subunit.^{9,10} Truncated "gp140" forms of Env that express gp120 and the ectodomain of gp41 assemble into oligomeric structures.⁴

Immunizations of rabbits with DNA vaccines expressing gp120 and gp140 forms of HIV-1 Env have demonstrated that these forms differ in their immunogenic potential.¹¹ DNA vaccines encoding gp120s from two series of patient isolates as well as from the T cell line-adapted strain, HXB-2, raised higher titers of antibody than the corresponding gp140s.¹¹ However, despite their low immunogenicity, the gp140-expressing plasmids were more effective at raising neutralizing antibodies, with different gp140s priming different patterns of low-titer neutralizing responses.¹² These results suggest that the gp140 oligomeric proteins had preserved critical immunological determinants for neutralization of HIV-1. However, the oligomeric gp140s raised only low-titer antibody responses. Therefore it is important to design Env structures that are more immunogenic than gp140, yet retain important neutralizing epitopes.

¹Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655.

²Division of Human Retrovirology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115.

³Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655.

⁴Department of Surgery and Center for AIDS Research, Duke University Medical Center, Durham, North Carolina 27708.

The gp120 glycoproteins from different HIV-1 strains exhibit regions of variability (V1-V5), some of which have been shown to exist as disulfide-linked loops. Prior studies have shown that deletion of the V1/2 and V3 regions of gp120 (dV123/gp120) unmasks the CD4-binding domain of Env while not disrupting the ability of gp120 to interact with gp41 and the CD4 receptor.¹³ In this article, Envs with deleted V1/2 and V3 domains were tested for their ability to improve the immunogenicity of Env. It was hoped that exposure of the CD4-binding domain might lead to the production of higher titers of neutralizing antibodies.

Vaccine plasmids encoding either variable loop-containing or loop-deleted forms of Env were prepared for the gp120, gp140, and gp160 forms of Env (Fig. 1). Variable loop-deleted *env* gene sequences were subcloned from the expression plasmid pSVIIIenv.¹³ The deletions encompassed amino acids 121 to 203 (V1/V2 domain) and amino acids 297 to 329 (V3 domain). A three-amino acid sequence (Gly-Ala-Gly) replaced each of the two deletions. Vaccine plasmids that expressed the gp120 and gp140 forms of the deleted Env (dV123.gp120 and dV123.gp140, respectively) were amplified from pSVIIIenv by polymerase chain reaction (PCR) and cloned in frame with the

TABLE 1. HIV-1 ENV EXPRESSION IN DNA-TRANSFECTED COS CELLS^a

	Total Env (ng/transfection)	Percentage in supernatant
Regular Env		
gp120	520	94
gp140	263	82
gp160	439	23
dV123/Env		
gp120	6795	89
gp140	1257	67
gp160	829	61

^aExpression of plasmids was tested in transiently transfected COS cells as previously reported.¹¹ Transfections used 1 to 3 μ g of a test DNA, 100 ng of the hGH-expressing plasmid (used as an internal transfection control), 10 μ l of lipofectAMINE (Life Technologies, Gaithersburg, MD), and $\sim 5 \times 10^5$ subconfluent COS cells. At 48 to 72 hr posttransfection, supernatants were harvested. The cells were detached from plates and lysed with 1% Triton X-100 buffer. Antigen capture ELISAs were used to determine the concentrations of Env. The amount of Env expression was calculated from the standard curve generated with the purified Env protein.

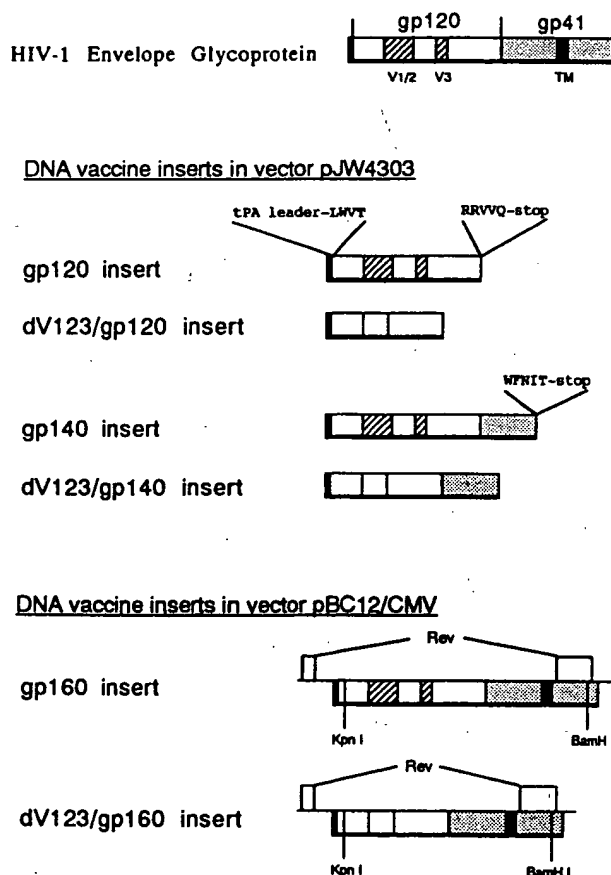


FIG. 1. Design and cloning of HIV-1 Env-expressing DNA vaccine inserts. tPA, tissue plasminogen activator leader sequences. pJW4303 expression vector¹⁴ was used for gp120- and gp140-expressing inserts and pBC12/CMV expression vector¹⁵ was used for gp160-expressing inserts. Amino acid sequences generated by the PCR primers at the junction of cloning sites are shown with single-letter codes.

tissue plasminogen activator (tPA) leader sequences in the vaccine expression vector pJW4303 (Fig. 1). pJW4303 is a eukaryotic expression vector that uses enhancer and promoter elements, including intron A, from the cytomegalovirus immediate-early promoter, and polyadenylation sequences from the bovine growth hormone (for details see Ref. 14). For both constructs, the common 5' primer JApr503 (5' GTCGCTC-CTCTAGATTGTGGGTCACAGTCTATTATGGGTACC) containing an *Xba*I site was used to clone *env* fragments in frame with the tPA leader sequences in pJW4303. For the gp120-expressing constructs, the 3' primer JApr504 (5' GGTCGGATCCttaaCTGCACCACTCTTCTCTTTGCC) was used to produce the gp120 inserts with a stop codon (in lower case) at the junction of gp120 and gp41. For the gp140-expressing constructs another 3' primer, JApr502 (5' CGACG-GATCCttaaTGTTATGTCAAACCAATTCCAC), introduced a stop codon (in lower case) immediately preceding the transmembrane domain of gp41 (Fig. 1). For the gp160-expressing Env, the *Kpn*I-*Bam*HI fragment of pSVIIIenv was substituted for the corresponding fragment in pNL4-3.env, which expresses a full-length Env and Rev (for details, see Ref. 15). Rev was not included in the gp120- and gp140-expressing plasmids as expression of Env with the tPA leader in pJW4303 allows Env expression without Rev.¹⁶

Expression of the Env-encoding constructs was examined in transiently transfected COS cells as previously reported.¹¹ Antigen capture ELISA, as well as Western blot analyses, revealed that the variable loop-deleted forms of Env were expressed at higher levels than the variable loop-containing forms (Table 1 and data not shown). The total levels of Env expression in COS cells and the fraction of Env in tissue culture supernatant were different among different forms of Env (Table 1). The dV123.env constructs had from 2-fold (dV123.gp160) to 10-fold (dV123.gp120) higher levels of expression than their variable loop-containing counterparts (Table 1). The antigen cap-

ture ELISA also revealed a higher proportion of the dV123.gp160 Env in the supernatant culture medium (61% of the total Env expression) than the variable region-containing gp160 (23% of the total Env expression). Western blot analysis of transfected COS cell lysates using monoclonal antibody Chessie 13-39.1 (Cat. no. 1209; NIH AIDS Repository),¹⁷ which recognizes a common sequence in each of the Envs, confirmed that the dV123 mutants were expressed at a higher level (data not shown).

The above-described Env-expressing DNA vaccines were administered to 8- to 10-week-old NZW rabbits by gene gun inoculation as previously described.¹¹ Plasmid vectors without *env* inserts were used as a negative control. Purified DNA plasmids were precipitated onto 0.95- μ m gold beads using spermidine in the presence of Ca^{2+} . All DNA inoculations were given to freshly shaved abdominal skin of NZW rabbits with an Accell[®] helium-driven gun (Geniva, Middleton, WI). Various immunization schedules were employed (see Fig. 2 caption for details). Rabbit sera were collected before the first immunization, 2 weeks after each immunization, and at additional time points to monitor the level and temporal pattern of anti-Env antibody responses.

The gp120, gp140, and gp160 forms of the normal HXB2 Env raised different patterns of anti-gp120 IgG responses. Tem-

poral antibody responses were screened by ELISA using recombinant gp120 (Intracel, Cambridge, MA) as the solid-phase antigen (Fig. 2). The gp120-expressing DNA was the most immunogenic. Anti-gp120 IgG responses were detected after one boost in each of the three immunized rabbits. The anti-gp120 responses showed reasonable persistence over a 16-week test period between the fourth and fifth immunizations. The gp140-expressing DNA vaccine was less immunogenic, with detectable anti-gp120 antibody responses scoring in one rabbit after three immunizations, the second rabbit after four immunizations, and the third rabbit after five immunizations. The antibody levels in this group fell to baseline level during the 16-week period between the fourth and fifth DNA immunizations. The fifth DNA immunization, however, was effective in bringing the antibody responses back to or above the previous levels, but it did not improve the persistence of the response. The gp160-expressing plasmid was the least immunogenic, with only one of the three rabbits having a significant response.

Deletion of the V1/2 and V3 domains substantially improved the immunogenicity of the gp140- and gp160-expressing vaccine constructs (Fig. 3). Responses were again measured against recombinant gp120. One rabbit immunized with the dV123.gp140-expressing DNA had anti-gp120 IgG responses

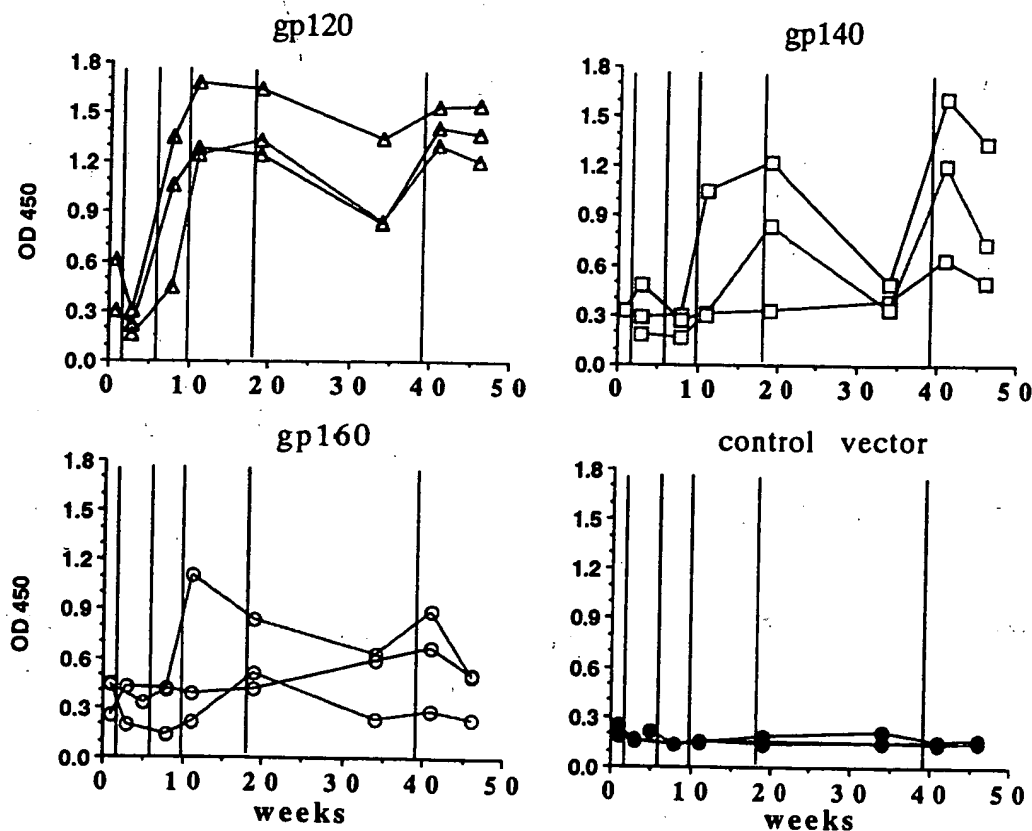


FIG. 2. Analysis of the ability of the variable loop-containing forms of Env to raise antibody in NZW rabbits. Recombinant variable loop-containing gp120 (Intracel, Inc.) was used as the coating antigen for ELISAs. Gene gun inoculations are indicated as vertical lines. Groups of three female rabbits were immunized with DNA vaccines using an Accell[®] helium-driven gene gun. Each curve represents one testing rabbit. For each inoculation, every rabbit received 36 shots of 0.25 μ g DNA each. pJW4303 vector without *env* insert was used as a negative control plasmid.

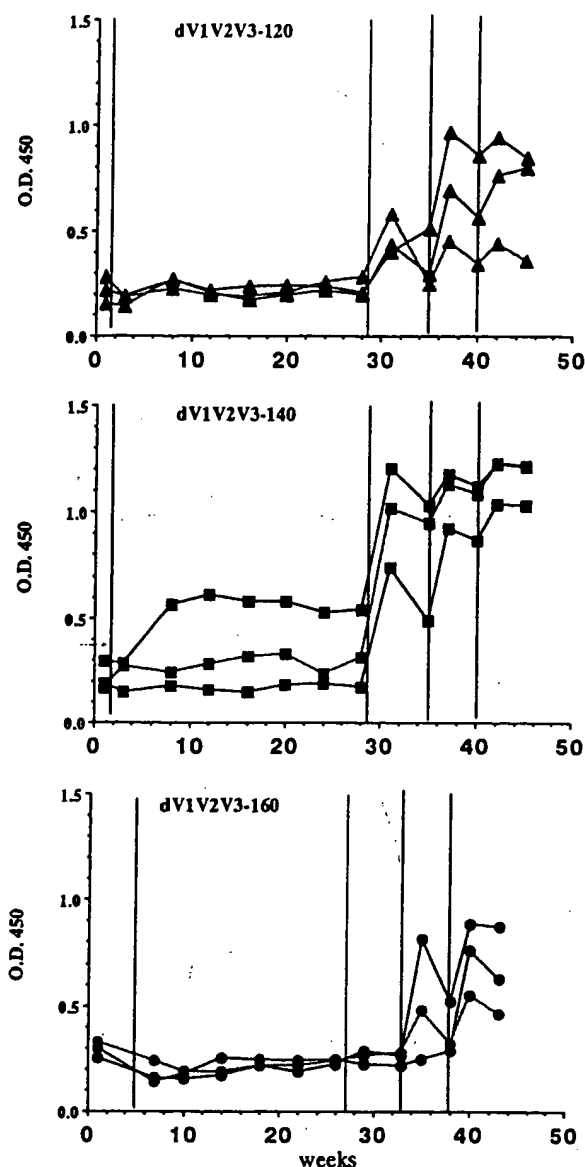


FIG. 3. Analysis of the ability of the variable loop-deleted forms of Env to raise antibody in NZW rabbits. Recombinant variable loop containing gp120 (Intracel) was used as the coating antigen for ELISAs. Gene gun inoculations are indicated as vertical lines. Groups of three female rabbits were immunized with DNA vaccines using an Accell helium-driven gene gun. Each curve represents one rabbit. For each inoculation, every rabbit received 36 shots of 0.25 μ g DNA.

after only one immunization and all three rabbits in this group had detectable responses after two immunizations. Two rabbits immunized with dV123.gp160 DNA vaccines had detectable anti-gp120 IgG responses after three immunizations and the third rabbit seroconverted after the fourth immunization. In contrast to the improved immunogenicity of the variable region-deleted gp140 and gp160 forms of Env, deletion of V1/2 and V3 appeared to reduce the immunogenicity of the gp120 form. Rabbits receiving the dV123.gp120-expressing DNA had lower titers of antibody than the rabbits that received the dV123.gp140 DNA (Fig. 3).

Deletion of the V1/2 and V3 domains exposed additional immunological determinants on Env. When rabbit sera were further tested in a quantitative ELISA using the recombinant dV123.gp120 protein as the coating antigen, all of the rabbits immunized with the dV123.Env-expressing DNA vaccines (R76–R84) had higher IgG responses against the mutant antigen than the wild-type gp120 antigen (Table 2). In contrast, the sera raised by the variable loop-containing Envs (R14–R19 and R104–R106) contained much lower activity for the variable region-deleted gp120. Thus, the deletion of the V1/2 and V3 regions appears to expose or create epitopes that were recognized by sera from dV123.Env-immunized animals, but not by sera from rabbits immunized with variable loop-containing forms of Env. These quantitative assays also revealed that among the three deletion constructs, the dV123.gp140-expressing DNA vaccine raised the highest titer antibody responses against both the regular gp120 and the dV123.gp120 antigens (Table 2).

When compared with the DNA expressing the variable loop-containing gp120 (R14–R16), the dV123.gp120-expressing DNA (R76–R78) raised lower titers of antibody for the vari-

TABLE 2. ELISA AND NEUTRALIZING TITERS OF DNA VACCINE-INDUCED RABBIT SERA*

DNA vaccine	Animal	Peak ELISA (μ g/ml)		Neutralization (IIIB)
		Regular gp120	dV123/gp120	
gp120	R14	8.30	1.10	1:31
	R15	6.30	1.94	1:39
	R16	5.70	1.81	1:23
gp140	R17	7.00	0.02	<1:10
	R18	3.30	0.06	1:28
	R19	1.60	0.02	
gp160	R104	0.01	0.02	<1:10
	R105	0.12	0.49	<1:10
	R106	0.01	0.04	<1:10
dV123/gp120	R76	0.34	4.32	<1:10
	R77	0.16	1.04	<1:10
	R78	0.10	0.49	
dV123/gp140	R79	2.07	59.88	<1:10
	R80	0.42	7.89	<1:10
	R81	2.57	18.99	
dV123/gp160	R82	0.35	14.19	<1:10
	R83	0.15	2.90	<1:10
	R84	0.06	0.23	

*ELISA values are for the peak antibody levels induced by the DNA immunizations. Quantitative anti-gp120 IgG levels were determined as previously described.¹² A standard curve was constructed using sera pooled from rabbits immunized with a plasmid expressing HXB-2 gp120, whose anti-Env IgG concentrations had been approximated. All samples and dilutions were assayed in duplicate wells, and a standard curve was included on each ELISA plate. Blank values were subtracted, standard curves were fitted using four-parameter equations, and sample concentrations were interpolated, as micrograms per milliliter of serum, using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA). The antibody-mediated neutralization of HIV-1 IIIB with rabbit sera at peak ELISA titers was measured in an MT-2 cell-killing assay as described previously.^{11,18}

able loop-containing gp120 antigen but higher titers of antibody for the variable loop-deleted gp120 (Table 2). This suggests that the removal of the V1/2 and V3 variable domains on the gp120 form of Env results in the loss of immunogenic determinants as well as the unmasking/creation of new determinants in gp120.

Differences in the titers of antibody raised by the different forms of Env could also have been affected by differences in the level of expression, or the extent of secretion, of a DNA-expressed immunogen (see Table 1). All of the variable loop-deleted forms of Env underwent better expression than the variable loop-containing forms of Env. The variable loop-deleted form of gp160 also underwent more shedding than the variable loop-containing form of gp160. Because of the marked differences in the reactivity of the raised sera for variable loop-containing and deleted forms of gp120 (Table 2), we consider it likely that exposure or creation of new epitopes by the variable loop deletions played a more important role in determining immunogenicity than differences in the levels of expression and shedding (Table 2).

Disappointingly, although deletion of the variable regions increased the titers of ELISA antibody raised by the gp140 and gp160 forms of Env, these structural changes reduced the ability of Env-expressing DNAs to raise neutralizing antibody (Table 2). Both the gp120 and gp140 forms of the variable loop-containing Envs raised neutralizing antibody, none of the variable region-deleted Envs raised detectable levels of neutralizing antibody. Thus, removal of the variable loops to achieve better exposure of the CD4 binding domain did not improve the elicitation of neutralizing antibodies. Given the fact that V1/2/3 removal decreased the ability of Env to raise neutralizing antibodies, most of the neutralizing antibodies raised by the intact envelope glycoproteins may be against the variable loops. Additional work will be required to determine optimal means to elicit neutralizing antibodies against conserved HIV-1 envelope glycoprotein determinants.

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Address reprint requests to:

Shan Lu

Department of Medicine

University of Massachusetts Medical Center

55 Lake Avenue, North

Worcester, Massachusetts 01655